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Master thesis

Development and biological validation of a patient-derived multicellular Synovia-on-Chip for precision medicine applications in Rheumatoid Arthritis

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SUMMARY

Rheumatoid arthritis (RA) is a chronic autoimmune disease characterized by persistent synovial inflammation, progressive joint destruction and marked heterogeneity in disease progression and therapeutic response. Despite the availability of several therapeutic options, treatment selection still relies largely on a trial-and-error approach, often resulting in suboptimal clinical outcomes. In this context, the development of *in vitro* models capable of reproducing patient-specific disease mechanisms is essential to advance precision medicine strategies. The aim of this thesis was to develop and biologically validate a multicellular Synovia-on-Chip (SoC) platform able to recapitulate key features of the RA joint microenvironment. The system integrates fibroblast-like synoviocytes (FLS) isolated from RA patients, which were also further differentiated into chondrocytes and osteoblasts to generate an autologous osteochondral compartment, together with vascular units formed by human umbilical vein endothelial cells (HUVECs). Prior to SoC assembly, different culture media were evaluated to identify the most suitable conditions for maintaining cell viability while supporting the metabolic requirements of all cellular compartments. Cells were then encapsulated within hydrogel-based matrices and organized into distinct microfluidic compartments, enabling controlled co-culture under dynamic perfusion. Viability, tissue organization and biological responses were assessed under both spontaneous and inflammatory conditions (IL-1β, IL-6, TNF-α) through Live/Dead assay and 37-plex cytokine analysis. The results confirmed the successful differentiation of FLS into both osteoblasts and chondrocytes and identified a 1:1 mixture of EGM and DMEM/F12 as the optimal culture medium for the SoC. High cell viability was maintained across all compartments under both spontaneous and inflammatory conditions, while exposure to pro-inflammatory cytokines induced morphological changes consistent with cellular activation and tissue remodelling. Specifically, FLS exhibited a more elongated phenotype whereas osteoblasts and chondrocytes showed a more organized spatial distribution, without compromising overall viability. Cytokine profiling further demonstrated a robust inflammatory response, characterized by increased secretion of mediators involved in bone remodelling (IL-34, IL-20), matrix degradation (MMP-1 and MMP-2), and FLS activation (LIGHT and IL-32).

Overall, these findings demonstrate that the RA-SoC is biologically responsive to pro-inflammatory stimuli and capable of reproducing key pathological processes associated with RA. This platform represents a promising patient-specific tool for studying disease mechanisms and evaluating therapeutic responses, providing a foundation for future precision medicine and clinical trial-on-chip applications in RA.

SOMMARIO

L'artrite reumatoide (AR) è una malattia autoimmune cronica caratterizzata da infiammazione sinoviale persistente, progressiva distruzione articolare e marcata eterogeneità sia nell'evoluzione clinica sia nella risposta ai trattamenti. Nonostante la disponibilità di numerose opzioni terapeutiche, la scelta della terapia si basa prevalentemente su un approccio per tentativi ed errori, che può portare a risultati clinici non ottimali. In questo contesto, lo sviluppo di modelli *in vitro* in grado di riprodurre i meccanismi patologici specifici del paziente rappresenta un passaggio fondamentale verso la medicina di precisione.

L'obiettivo di questa tesi è stato lo sviluppo e la validazione biologica di una piattaforma multicellulare Synovia-on-Chip (SoC) in grado di ricapitolare le principali caratteristiche del microambiente articolare dell'AR. Il modello integra fibroblasti sinoviali derivati da pazienti affetti da AR, condrociti e osteoblasti differenziati partendo dai fibroblasti sinoviali per generare un compartimento osteocondrale autologo insieme a unità vascolari costituite da cellule endoteliali della vena ombelicale umana. Prima dell'assemblaggio della SoC, sono stati confrontati diversi terreni di coltura per individuare le condizioni più adatte a garantire la vitalità cellulare e a soddisfare le esigenze metaboliche dei diversi compartimenti cellulari. Successivamente, le cellule sono state incapsulate in matrici idrogeliche e organizzate in compartimenti microfluidici distinti, consentendo una co-coltura controllata in condizioni di perfusione dinamica. La vitalità cellulare, l'organizzazione tissutale e la risposta biologica sono state analizzate sia in condizioni spontanee sia in presenza di uno stimolo infiammatorio (IL-1β, IL-6, TNF-α) mediante saggio Live/Dead e analisi di 37 citochine.

I risultati hanno confermato il corretto differenziamento dei fibroblasti sinoviali in osteoblasti e in condrociti e hanno identificato una miscela di EGM e DMEM/F12 in rapporto 1:1 come terreno di coltura ottimale per la SoC. Le cellule hanno mantenuto un'elevata vitalità cellulare in tutti i compartimenti sia in condizioni spontanee sia infiammatorie, mentre l'esposizione alle citochine pro-infiammatorie ha indotto modificazioni morfologiche compatibili con processi di attivazione cellulare e rimodellamento tissutale. L'analisi del profilo citochinico ha inoltre dimostrato una robusta risposta infiammatoria, caratterizzata da un aumento della secrezione di mediatori coinvolti nel rimodellamento osseo (IL-34, IL-20), nella degradazione della matrice extracellulare (MMP-1 e MMP-2) e nell'attivazione dei fibroblasti (LIGHT e IL-32).

Nel complesso, questi risultati dimostrano che il modello SoC di AR risponde biologicamente agli stimoli pro-infiammatori ed è in grado di riprodurre i principali processi patologici associati alla patologia. Questa piattaforma rappresenta un promettente strumento paziente-specifico per lo studio

dei meccanismi della malattia e la valutazione della risposta terapeutica, ponendo le basi per future applicazioni della medicina di precisione e dei clinical trial-on-chip nel contesto dell'AR.

1. INTRODUCTION

1.1 RHEUMATOID ARTHRITIS (RA)

Rheumatoid arthritis (RA) is a chronic systemic inflammatory disease that primarily targets the synovial joints. It is the most prevalent systemic autoimmune disease among inflammatory rheumatic musculoskeletal disorders and is often challenging to manage, typically requiring lifelong therapy (Finckh et al., 2022). It is characterized by progressive and destructive arthropathy resulting from synovial inflammation and hyperplasia. These processes lead to cartilage and bone damage and may result in extra-articular manifestations such as cardiovascular, pulmonary, renal, gastrointestinal, neurological and hematological disorders, which contribute to increased morbidity and premature mortality (Jang et al., 2022; Figus et al., 2021).

RA is distributed worldwide, with an estimated prevalence ranging from 0.24% to 2%. Its incidence increases in individuals over 40 years of age and women are two to three times more likely to develop RA than men (Uke et al., 2025).

The clinical manifestations of RA depend on the involved joints and the stage of the disease. RA commonly presents as symmetric arthritis, leading to articular and periarticular manifestations such as joint swelling and tenderness, often accompanied by morning stiffness and impaired joint function. The most frequent presentation is an insidious onset of pain, although in about 25% of patients the onset may be acute or subacute. The pattern of presentation can be palindromic or monoarticular, along with other general symptoms such as fatigue, weight loss, fever and malaise (Grassi et al., 1998).

In addition to its clinical impact, RA is associated with a substantial socio-economic burden, including indirect costs related to loss of productivity and increased healthcare expenditures. Furthermore, limited accessibility to advanced therapies contributes to disparities in disease management and outcomes (Finckh et al., 2022).

The etiology of RA is still largely unknown; however, it is widely recognized as a multifactorial disease in which both genetic and environmental factors play an important role in its development. The overall heritability of RA is estimated at approximately 66%, with increased risk observed in first-degree relatives, reflecting a complex interplay between the genetic predisposition and shared environmental exposures (Gao et al., 2024). Genetic polymorphisms contribute to 30–60% of risk and currently more than 150 genetic loci have been associated with RA susceptibility. The strongest genetic association is linked to the inheritance of specific HLA-DRB1 alleles, in particular DR4 and DR1. These alleles contain a conserved five-amino-acids motif known as shared epitope, located in the antigen-binding groove of HLA-DR molecule and involved in the presentation of antigens to CD4+ T cells (Scherer et al., 2020).

Environmental factors also contribute to RA susceptibility. Among these, cigarette smoking has been identified as a major risk. Other factors, including diet and microbiome, may also influence disease onset and progression (Maisha et al., 2023).

Taken together, these findings support the concept that RA arises from a complex interplay between genetic susceptibility and environmental triggers, ultimately leading to a loss of immune tolerance reflected in the presence of circulating autoantibodies, leading to the classification of RA into seropositive and seronegative forms. Seropositive RA is characterized by the presence of autoantibodies such as rheumatoid factor (RF) and anti-citrullinated protein antibodies (ACPAs) in the serum, whereas seronegative RA lacks these markers. Susceptibility to ACPA-positive RA is strongly associated with the presence of HLA-DRB1 alleles encoding the shared epitope (Zamanpoor, 2019).

1.1.1 Immunopathogenesis of RA

The autoimmune pathogenesis of RA was first recognized through the identification of autoantibodies targeting the Fc portion of immunoglobulin G (IgG), referred to as RF (Weyand & Goronzy, 2021).

Increasing evidence suggests that RA originates from a systemic breakdown of immune tolerance occurring prior to the onset of synovial inflammation. In genetically predisposed individuals, this process results in the production of autoantibodies, directed against post-translationally modified proteins, including citrullinated and carbamylated antigens (Deane et al., 2010). Emerging studies indicate that this pre-clinical autoimmune activity often originates at mucosal sites, such as the lungs, oral cavity, and gut, where environmental triggers, such as smoking or dysbiosis, may initiate immune activation (Joshua et al., 2023; Zaiss et al., 2021). This pre-clinical autoimmune phase is sustained by T-cell-dependent B-cell responses and may persist for years before progressing to clinically evident disease (Deane et al., 2010).

Although this prolonged phase of asymptomatic autoimmunity occurs outside the joints, the disease subsequently localizes to the synovium when innate and adaptive immune cells infiltrate the synovial membrane (Weyand & Goronzy, 2021).

At this stage, RA is characterized by a complex interplay of cellular and molecular mechanisms, including the activation of immune cells, production of pro-inflammatory cytokines, and release of local growth factors, all of which contribute to synovial hyperplasia (**Figure 1**).

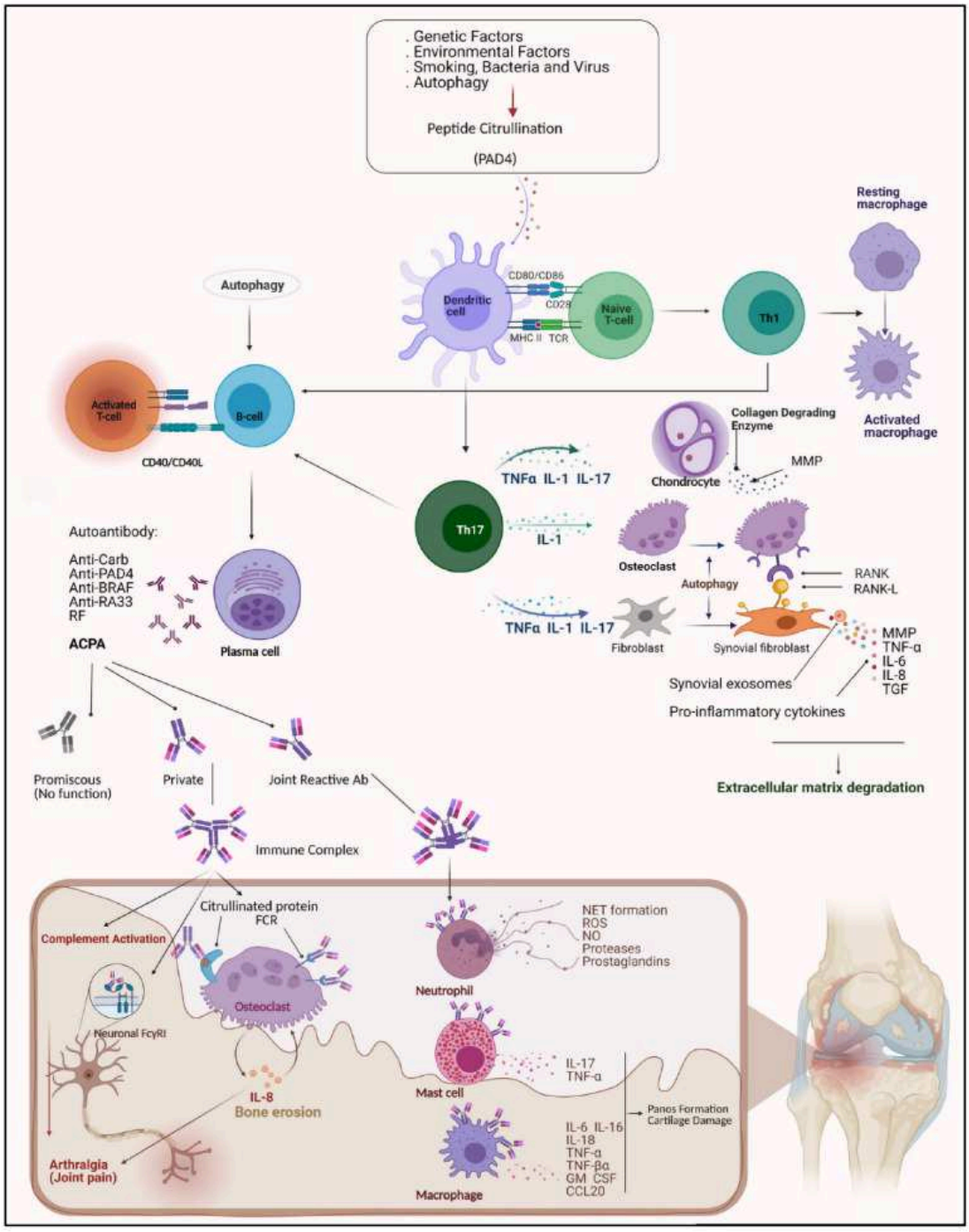

Figure 1: ***Immunopathogenesis of RA***. *Genetic and environmental factors promote citrullination of proteins, generating neoantigens that activate dendritic cells and T cells (Th1/Th17). These responses drive B-cell activation and production of autoantibodies (e.g., ACPA, RF), forming immune complexes that trigger complement activation and inflammation. Pro-inflammatory cytokines (TNF-α, IL-1, IL-17) activate macrophages, synovial fibroblasts, and osteoclasts, leading to cartilage degradation and bone erosion, which underlie joint damage in RA (Mueller et al., 2021).*

Immune cells infiltrating the synovial tissue drive the production of inflammatory and degradative mediators, ultimately leading to the degradation of the extracellular matrix (ECM) in cartilage and bone (Jahid et al., 2023). Building on the genetic susceptibility associated with HLA-DRB1 alleles,

CD4+ T lymphocytes play a central role in initiating and sustaining autoreactive immune responses in RA, following their recruitment to inflamed synovial tissue (Jang et al., 2022; Mellado, 2015). Among T-cell subsets, T helper 17 (Th17) cells are key drivers of disease progression, as they produce interleukin-17 (IL-17), a potent pro-inflammatory cytokine. IL-17 amplifies the inflammatory response by inducing the production of tumor necrosis factor-α (TNF-α), IL-1β, and IL-6 in cartilage, synovial cells, macrophages and bone cells (Onishi & Gaffen, 2010).

B cells play a central role in RA by producing autoantibodies such as RF and ACPAs, presenting antigens to T cells and secreting pro-inflammatory cytokines. Through these mechanisms, B cells help sustain chronic synovial inflammation and contribute to joint damage, acting as key drivers of the autoimmune response (Bugatti et al., 2014).

Although adaptive immunity (T and B cells) is central in RA pathogenesis, innate immune cells also play a key role in disease initiation and progression, shaping adaptive responses. Among these, macrophages are the most abundant in the synovium and contribute to disease progression through the secretion of pro-inflammatory cytokines (TNF-α, IL-1β and IL-6), chemokines (CCL-2 and IL-8) and matrix metalloproteinases (MMP-3 and MMP-12). Pro-inflammatory M1 macrophages promote joint damage, whereas anti-inflammatory M2 macrophages regulate inflammation, support tissue repair, and promote angiogenesis (Edilova et al., 2021).

The synovium, composed of macrophage-like synovial cells and fibroblast-like synoviocytes (FLS), undergoes profound structural changes in RA, transforming into a hyperplastic and invasive tissue rich in immunocompetent cells. M1 macrophages drive the activation of FLS, which induce the production of a wide range of mediators, including pro-inflammatory cytokines, prostanoids and matrix metalloproteinases (MMPs). These factors promote synovitis by recruiting additional cells to the joint and contributing to degradation of ECM. The progressive expansion of this inflamed synovial tissue leads to the formation of pannus, a hyperplastic structure located at the cartilage-bone interface. The pannus, composed mainly of macrophages, osteoclasts and invasive FLS behaves as a tumour-like structure that invades cartilage and promotes bone erosion (Bartok & Firestein, 2010). Angiogenesis is a hallmark of this process, promoted by FLS through the secretion of vascular endothelial growth factor (VEGF) that leads to endothelial cell activation and neovascularization within the inflamed synovium. Osteoclast activation is mediated by inflammatory cytokines and receptor activator of NF-κB ligand (RANKL) expressed by FLS, T cells or B cells, highlighting the central role of stromal-immune cell interactions in driving bone erosion in RA. FLS also directly contribute to cartilage destruction through the release of degradative enzymes such as MMPs and cathepsins. In parallel, chondrocytes actively participate in cartilage degradation by acquiring a catabolic phenotype in response to pro-inflammatory cytokines, further amplifying extracellular

matrix breakdown through the secretion of degradative enzymes (Meednu et al., 2016; Noss & Brenner, 2008).

Overall, RA pathogenesis is not solely driven by immune cells, but rather by a complex interplay between immune and resident joint cells, including FLS, chondrocytes, osteoclasts and endothelial cells, which collectively drive chronic inflammation and tissue destruction (Noss & Brenner, 2008).

1.1.2 Diagnosis and therapeutic strategies

Given the heterogeneous and complex nature of RA, characterized by diverse clinical presentations, diagnosis relies on a comprehensive and individualized assessment performed by a rheumatologist, integrating clinical manifestations, serological assays and imaging findings (Zamanpoor, 2019).

The American College of Rheumatology (ACR) in 1987 proposed classification criteria to identify established RA; patients are classified as such whether they have four of the seven disease features: morning stiffness in joints, arthritis in three or more joint areas, arthritis in hand joints, symmetrical arthritis, rheumatoid nodules, presence of RF in the serum, and radiographic evidence of joint erosion. However, these criteria showed limited sensitivity in detecting early-stage RA; the ACR and the European League Against Rheumatism (EULAR) introduced updated classification criteria in 2010. These are based on a scoring system that incorporates joint involvement, serological markers such as RF and ACPAs, acute-phase reactants, and symptoms duration, allowing for earlier and more accurate identification of the disease (Kourilovitch et al., 2014; Zamanpoor, 2019).

Once the diagnosis is established, the management of RA aims to control inflammation, relieve symptoms, and prevent structural joint damage through pharmacological interventions. Non-steroidal anti-inflammatory drugs (NSAIDs) are commonly used as first-line agents for symptomatic relief. They are effective in reducing pain, swelling, and morning stiffness, but do not modify disease progression. NSAIDs, including drugs such as ibuprofen, naproxen, celecoxib and acetylsalicylate, inhibit cyclooxygenase (COX) enzymes, thereby decreasing the synthesis of prostaglandins, key mediators of inflammation (Jahid et al., 2023).

Due to the limited disease-modifying effects of NSAIDs and corticosteroids, disease-modifying anti-rheumatic drugs (DMARDs) represent the cornerstone of RA treatment. DMARDs are immunomodulatory agents aimed at slowing disease progression and reducing inflammatory activity. They are classified into conventional synthetic DMARDs (csDMARDs), biologic DMARDs (bDMARDs) and targeted synthetic DMARDs (tsDMARDs) (Huang et al., 2021).

Among csDMARDs, methotrexate (MTX), a folate analogue leading to the inhibition of nucleotide synthesis and metabolism, represents the first-line therapeutic option for RA, owing to its well-established efficacy, relatively low cost, quick onset of action and ease of administration. However approximately 50% of patients either experience adverse effects or fail to achieve an optimal clinical response (Jahid et al., 2023; Prasad et al., 2023).

bDMARDs, usually prescribed after failure of csDMARDs, are biologic agents, most commonly monoclonal antibodies or receptor fusion proteins, that modulate immune responses by targeting B/T cell activation or pro-inflammatory cytokines. A major subclass includes TNF-alpha inhibitors such as Infliximab, Adalimumab, Certolizumab, Etanercept which block the interaction between TNF-alpha and its receptor, thereby preventing down-stream inflammatory signaling (Kumar & Banik, 2013).

Another important therapeutic target in RA is the Janus kinase–signal transducer and activator of transcription (JAK-STAT) pathway, which mediates cytokine-driven inflammatory signaling downstream of IL-6, Interferon-gamma (IFN-γ) and Granulocytes-Macrophage Colony-Stimulating Factor (GM-CSF). tsDMARDs such as Tofacitinib, Baricitinib and Filgotinib inhibit this pathway by interfering with intracellular signal transduction (Fragoulis et al., 2019).

Despite the wide range of available therapies, the management of RA remains challenging due to a considerable portion of patients who do not achieve an adequate clinical response, estimated at approximately 40% after exposure to different therapeutic options. Importantly, currently there are no available biomarkers that can aid clinicians in choosing the appropriate therapeutic option for each patient. This often leads to a "trial-and-error" approach, which may result in unnecessary exposure to potentially toxic treatments, delayed disease control, and increased healthcare costs (Gudu et al., 2025). In this context, personalized medicine approaches are needed to guide therapeutic decisions and optimize treatment selection based on patient-specific characteristics (Tanaka, 2021).

To move towards a more predictive and personalized approach, innovative experimental tools such as Organ-on-Chip (OoC) technology have emerged.

1.2 ORGAN-ON-CHIP

1.2.1 Limitations of conventional *in vitro* and *in vivo* models

Traditionally, RA has been studied using a combination of *in vitro* and *in vivo* models. *In vitro* approaches are mainly based on simplified cell culture systems, including co-cultures. However, conventional two-dimensional (2D) cell culture models fail to reproduce the complex physiological

environment of human tissues, lacking critical biochemical, mechanical, and three-dimensional (3D) structural cues necessary to maintain proper cell function and phenotype. As a result, these models provide limited insight into the pathophysiology of RA and show poor clinical translatability. In parallel, animal models have been widely used to study RA, with murine models being the most common. Although they have provided valuable information on disease mechanisms and therapeutic targets, their translational relevance is limited by fundamental differences between human and animal immune systems, as well as by the artificial induction of disease, which does not fully reflect the complexity and heterogeneity of human RA (Cassotta, 2019). Compared to 2D culture systems, *in vitro* 3D tissue engineering approaches are particularly promising because they facilitate cell-cell and cell-matrix interactions, as well as cell proliferation, differentiation and migration. These approaches include scaffold-based systems, but also spheroids and organoids, which promote cell self-organization and better mimic the structural and functional properties of native tissues. In particular, the presence of an ECM-like environment enhances the structural organization and relevant cellular functions compared to 2D cultures. However, despite these advantages, conventional 3D models still present some limitations, including limited reproducibility and insufficient control over nutrients, oxygen diffusion and waste removal within the constructs. In this context, microfluidic technologies have emerged as a valuable tool to overcome these challenges by enabling controlled medium perfusion and the application of mechanical stimuli (Badillo-Mata et al., 2022).

To more closely mimic the features of the joint in RA pathogenesis, all relevant tissue components, including the synovial membrane, as well as chondrogenic and osteogenic compartments, should be incorporated into a 3D *in vitro* approach (Damerau & Gaber, 2020). To combine the advantages of 3D models with the possibility of integrating multiple tissue types, OoC platforms have been developed, enabling the co-culture of different cellular compartments with a controlled microfluidic environment (Li et al., 2023).

1.2.2 Organ-on-Chip technology

Many diseases, including RA, are characterized by the lack of clinically relevant experimental models. In this context, OoC systems have been developed to overcome the limitations of other preclinical approaches. OoC platforms can be described as miniaturized and micro-engineered systems in which cells are cultured within a 3D environment under controlled flow conditions, allowing them to better resemble their *in vivo* counterparts. These systems incorporate both cellular and extracellular components of native tissues enabling the simulation of tissue-tissue crosstalk under physiological and pathological conditions (Li et al., 2023). A key feature of OoC systems is the

integration of microfluidic technology, which enables precise control over the cellular microenvironment. In particular, the dynamic nature of microfluidic systems allows the application of controlled biophysical and biochemical stimuli, playing a key role in regulating cell morphology, proliferation and gene expression. In addition, interconnected micro-channels facilitate the communication between different cellular compartments, allowing the study of tissue–tissue interactions that are critical in disease progression (Piluso et al., 2019).

Building on these advantages, OoC technologies have evolved toward increasingly complex multi-compartment systems capable of reproducing physiologically relevant tissue interactions (Wysoczański et al., 2024). These systems have been applied to model several organs, including liver (Nakao et al., 2011), lung (Douville et al., 2011), heart (Grosberg et al., 2011), blood vessel (Song & Munn, 2011) and muscle (Grosberg et al., 2012); in addition, OoC platforms have been used to recapitulate some pathological conditions such as cancer, neurodegenerative disorders and rare diseases (Celikkin et al., 2021).

Overall, OoC technologies address key limitations of conventional models by providing more predictive platforms for drug testing, improving the understanding of disease mechanisms, and supporting the development of more efficient and cost-effective drug discovery process. Moreover, their ability to incorporate human cells from different donors enables the investigation of inter-individual variability, a critical aspect for improving translational relevance in pharmacological studies (Celikkin et al., 2021). In this context, OoC platforms are increasingly recognized as valuable tools for toxicity assessment, biomarker identification and drug screening, and their application is progressively expanding toward more personalized approaches in biomedical research (Paul et al., 2010).

Overall, these features make OoC platforms particularly suitable for reproducing the complexity of joint tissues involved in RA pathogenesis (Pye et al., 2025).

1.2.3 Joint-on-Chip models

The synovial joint is a highly complex multi-tissue system comprising cartilage, bone and synovial membrane, each performing specific functions and tightly interacting with the others. Its complex cellular and molecular interplay makes it challenging to fully recapitulate joint physiology *in vitro* models (Piluso et al., 2019). OoC devices offer a promising strategy to incorporate joint-relevant tissues, in order to better mimic the native articular microenvironment (Paggi et al., 2022). In recent years, several OoC platforms have been developed to reproduce specific aspects of the synovial microenvironment in RA. Early approaches focused on 3D synovium-on-chip (SoC) systems based

on FLS organoids, enabling the investigation of inflammatory synovial tissue responses (Rothbauer et al., 2020). Building on these models, more advanced platforms have incorporated chondral and synovial organoids-on-a-chip to study the reciprocal inflammatory cross-talk (Rothbauer et al., 2021). More recently, RA-FLS were combined with M1 macrophages and human umbilical vein endothelial cells (HUVECs) on a microfluidic chip to recreate a vascularized and immunologically relevant SoC model for predicting drug responses (Diao et al., 2025). Despite these advances, most current models remain limited to specific compartments and do not fully capture the complex multi-tissue interactions that characterize the rheumatoid joint. Moreover, these models are not fully autologous, rendering them not entirely reliable in predicting patient-specific drug responses, and thus not suitable for replacing conventional clinical trials.

1.3 PRECISION MEDICINE

Precision medicine is a highly individualized and tailored approach to patient management. In RA, a complex and multifactorial disease, patients exhibit marked heterogeneity, which can hinder diagnosis, prognosis and treatment selection. Therefore, the pursuit of precision medicine in RA has become a major focus of current research (Aletaha, 2020). In this context, autologous organ-on-chip (aOoC) models have emerged as a new tool to study disease mechanisms, discover biomarkers and test therapeutic candidates. These systems rely on the incorporation of patient-derived cells into OoC platforms to preserve genomic, epigenomic and phenotypic traits of the donor (Bisconti et al., 2025). The use of primary cells, which can be isolated from human biopsies or discarded tissues, enables the generation of pharmacologically reliable data on drug response and toxicity, as they more accurately recapitulate human physiological conditions compared to immortalized cell lines (Jodat et al., 2019). This approach allows the evaluation of patient-specific drug responses and disease mechanisms, providing a more accurate representation of individual variability compared to conventional *in vitro* and *in vivo* models (**Figure 2**).

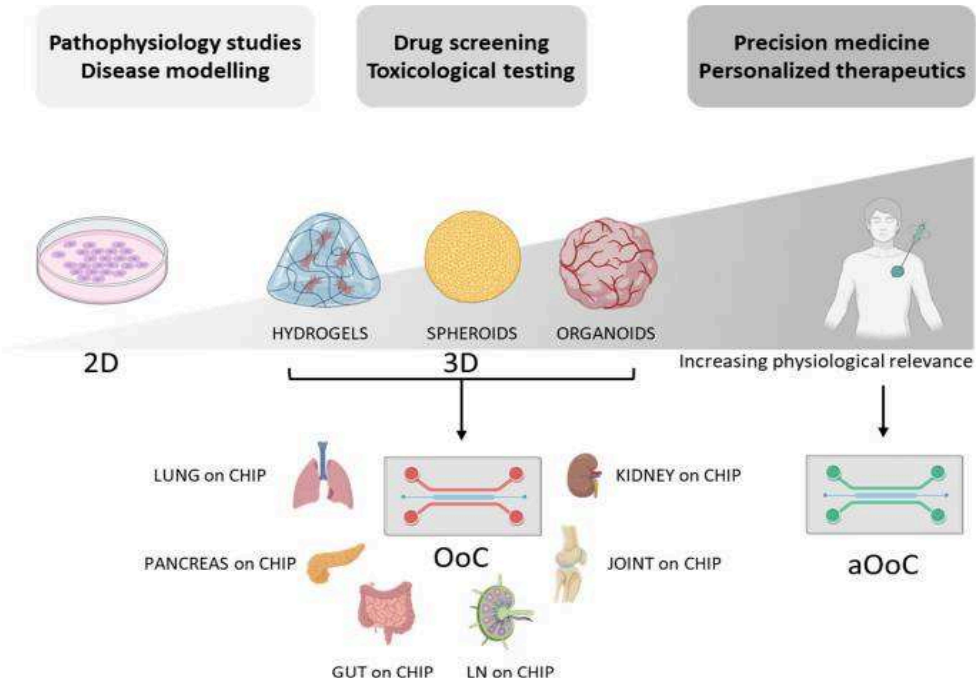

Figure 2*: **Evolution of in vitro models**. Advancements from conventional 2D cultures to advanced 3D systems and organ-on-chip (OoC) technologies, highlighting the progressive increase in physiological relevance. In particular, autologous OoC (aOoC) technologies enable more accurate disease modelling, drug screening and the development of personalized therapeutic strategies (Bisconti et al., 2025).*

The integration of patient-derived tissues within microfluidic systems also support the development of a clinical trial-on-chip (CToC) approach, in which multiple therapeutic strategies can be tested on patient-specific microtissues prior to clinical administration, in order to reduce the traditional trial-and-error process and to improve therapeutic outcomes (Ayuso et al., 2022).

The potential of patient-derived OoC technologies has already been demonstrated in several fields of precision medicine, particularly in oncology. Patient-derived tumor-on-chip models have been successfully developed using cells isolated from pancreatic ductal adenocarcinoma allowing the individualized study of cancer biology and drug response (Adnan et al., 2026), or from esophageal adenocarcinoma to predict patients' responses to chemotherapy and to test alternative therapies (Pal et al., 2025). Similar approaches have been applied to inflammatory bowel diseases, where gut-on-chip platforms incorporating patient-derived intestinal cells have been used to investigate disease mechanisms and evaluate personalized therapeutic strategies (Beaurivage et al., 2020).

The application of aOoC is particularly relevant in RA for several reasons. First, RA pathogenesis is multifactorial and involves complex interactions across multiple tissues. Second, responses to current therapies vary widely between patients. Third, robust biomarkers for patient stratification remain limited. In this context, biomarker-based patient stratification could be crucial to better understand disease heterogeneity and to define patient-specific pathological mechanisms. Moreover, the

validation of these patient subgroup-specific models will benefit from ongoing advances in disease modelling technologies and an improved understanding of underlying disease mechanisms (Bisconti et al., 2025; Li et al., 2023). However, biomarker discovery and validation, which can then lead to guideline updates and new patient re-stratification can be a lengthy process, thus not offering an immediate solution for RA treatment (Soyfoo & Sarrand, 2026).

Conceptually, using aOoC based on patient-derived cells enables the recreation of a physiologically relevant microenvironment that closely mimics individual human organ function for disease modelling and drug testing. This approach enables the development of more accurate and representative OoC models, thus providing a tool to perform a personalized CToC able to predict optimal therapies for each patient. Compared to conventional "one-size-fits-all" systems, RA-patient-derived SoC platforms may accelerate treatment selection, reduce adverse effects, and lower the socio-economic burden of the disease. Beyond RA, this approach is also adaptable to other conditions, such as osteoarthritis, and may support both drug development and disease modelling across indications (Bisconti et al., 2025).

2. OBJECTIVE OF THE THESIS

The main goal of this thesis was to assemble a complete RA-SoC and perform its biological validation under spontaneous and inflammatory conditions.

The objective of this thesis aligns with the goals of the European project "From Pathobiology to Synovia on Chip: Driving Rheumatoid Arthritis to the Precision Medicine Goal" (FLAMIN-GO) that has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement Number 953121.

The FLAMIN-GO project is designed to advance personalized care in RA through the development of a SoC platform. This approach targets several key objectives: identifying the most effective approved therapy for individual patients to optimize efficacy while reducing adverse effects; enabling the rapid identification and validation of novel therapeutic targets; and supporting future drug development pathways.

At the core of the project is a multi-compartment microfluidic system engineered to support three-dimensional culture and dynamic perfusion of all major joint components. This includes synovial tissue, synovial fluid, vascular structures, immune cells, cartilage, and bone. By replicating the biological complexity of an arthritic joint using patient-derived biopsy material, FLAMIN-GO seeks to establish a next-generation, patient-specific SoC platform, with the potential to evolve into a fully personalized CToC framework.

The work developed during this thesis is intended to serve as foundation for the establishment of a fully competent SoC, sustaining the future incorporation of the immune system and subsequent assessment of the efficacy of different drugs in a patient-centred personalized approach.

3. MATERIALS AND METHODS

3.1 CELL CULTURE

3.1.1 FLS extraction from synovial biopsies of RA patients

FLS were isolated from synovial biopsies of RA patients, provided by Queen Mary University of London (QMUL), by enzymatic digestion of the tissue using a solution of collagenase type I (1 mg/mL) (Sigma-Aldrich®, Germany) in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, USA). The solution was filtered using 0,22 µm filter to sterilize it prior to use.

The synovial tissue sample was placed into a Petri dish and minced into small fragments using a sterile scalpel. The tissue pieces were transferred into a 15 mL tube containing the digestion solution and incubated at 37 °C for 30 minutes, under gentle agitation to facilitate enzymatic digestion.

Following the digestion, tissue fragments were collected using a 2 mL serological pipette and transferred into a T75 flask. The fragments were evenly distributed and allowed to adhere to the flask surface for 40 minutes without media. Subsequently, 30 mL of Dulbecco's Modified Eagle Medium/Nutrient Mixture F12 (DMEM/F12) (Gibco, USA) supplemented with 10% of Fetal Bovine Serum (FBS) (Gibco, USA), 0.1 % Gentamicin (Gibco, USA), and 1% Penicillin-Streptomycin (Corning, USA) were gently added to avoid the detachment of the tissue pieces; they were maintained for 4/5 days to allow cell outgrowth from the tissue fragments. Then, the tissue was carefully removed and placed in a new T75 flask without media, in order to maximize cell retrieval. Again, after 40 minutes complete DMEM/F12 was added and after 4/5 days tissue fragments were discarded. During cell expansion, media was renewed every 2 days.

3.1.2 FLS culture

FLS derived from synovial biopsies of RA patients were expanded and cultured in T175 flasks using 20 mL of complete DMEM/ F12.

At approximately 90% confluence, cells were detached for further expansion and differentiation. The culture medium was removed and the flask was washed with phosphate-buffered saline (PBS) 1X. Subsequently, 3 mL of Trypsin-EDTA 1X were added and cells were incubated at 37 °C for 5 minutes until detachment. Trypsin activity was neutralized by adding 10 ml of complete DMEM/F12.

The cell suspension was collected in a 15 mL tube and centrifuged at 1500 rpm for 5 minutes. The supernatant was discarded and the pellet was resuspended in 1 ml of DMEM/F12 for cell counting. Cells were diluted 1:10 with Trypan Blue (Gibco, USA) and counted using a Bürker chamber. Following counting, FLS were seeded for expansion and subsequent differentiation into chondrocytes and osteoblasts using specific differentiation media. For the subsequent experiments, FLS in passages 3 to 6 were used, to ensure phenotype stability, as previously optimized in the lab.

3.1.3 Differentiation of FLS into chondrocytes and osteoblasts

Cells were divided into three T75 flasks to obtain 1 T75 of FLS, 1 T75 of chondrocytes and 1 T75 of osteoblasts. FLS were maintained in culture with DMEM/F12, while osteogenic and chondrogenic differentiation were induced using specific media.

OSTEOGENIC MEDIUM was prepared using complete DMEM Low Glucose (1g/L) (Gibco, USA), supplemented with ascorbic acid-2-phosphate (AA2P) (50 µg/mL), β-glycerophosphate (BGP) (5 mM) and dexamethasone (Dexa) (10 nM) (all from Sigma-Aldrich®, Germany).

CHONDROGENIC MEDIUM was prepared using complete DMEM High Glucose (4,5 g/L) (Gibco, USA), supplemented with AA2P (50 µg/mL), insulin-transferrin-selenium (ITS) (1%) (Corning, USA), Dexa (100 nM) and non-essential amino acids (NNEAA) (1%) (Gibco, USA). Then, transforming growth factor (TGF-β) (10 ng/mL) (PeproTech, USA) was added directly in the flask instead of mixing it with the medium.

In each T75 flask 10 mL of differentiation medium were added and replaced twice per week for 2 weeks.

After this period, cells were detached following the same procedure described above for FLS, with the exception that trypsin incubation was extended to 20 min at 37 °C, due to the increased extracellular matrix production by differentiated cells.

3.1.4 HUVECs culture

Commercial HUVECs were cultured in T175 flasks previously coated with 10 mL of 0.2% gelatin (Sigma Aldrich®, Germany). Cells were maintained in 20 mL of complete Endothelial Cell Growth Medium 2 (EGM-2 supplemented with Growth Medium 2 SupplementMix) (PromoCell GmbH, Germany). At approximately 90% confluence cells were detached and expanded following the same procedure described for FLS.

3.2 HYDROGEL PREPARATION

3.2.1 GelMA and LAP preparation

Gelatin methacryloyl (GelMA) is a gelatin derivative functionalized with methacrylamide and methacrylate groups. GelMA hydrogels are widely used in biomedical applications due to their biocompatibility and tunable mechanical properties. Upon exposure to UV light in the presence of a photoinitiator, GelMA undergoes free-radical photo polymerization, forming covalently cross-linked

hydrogel network. Lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP) is a commercially available water-soluble photointiator commonly used for this purpose (Yue et al., 2015).

A sterile 20% GelMA solution was prepared by dissolving freeze-lyophilized GelMA, provided by AO Foundation (FLAMIN-GO partners in Davos, Switzerland) in 10% FBS in PBS 1X. In parallel, a 2% LAP (Sigma Aldrich®, Germany) solution was prepared by dissolving the powder in 10% FBS in PBS. Both solutions were stirred at 60 °C for 1 hour with a magnetic stir bar inside until complete dissolution.

After that, they were diluted in complete medium to reach the desired concentration to encapsulate cells:

- For FLS: GelMA 5% + LAP 0.3%
- For osteoblasts and chondrocytes: GelMA 8% + LAP 0.5%

3.2.2 Fibrin-Gelatin-Transglutaminase (FGT) hydrogel preparation

The fibrin-gelatin-transglutaminase (FGT) hydrogel was used to encapsulate HUVECs and obtain vessel-like structures. The hydrogel is composed by a solution of fibrinogen from human plasma (10 mg/mL) (Sigma-Aldrich®, Germany), $CaCl_2$ (2.5 mM) (Sigma-Aldrich®, Germany) and transglutaminase (2 mg/mL) (Bindly ®, Spain). Gelatin from porcine skin 300 type A (7,5%) (Sigma-Aldrich®, Germany) was then added as the final component. All components were pre-warmed at 37 °C before mixing.

FGT hydrogel formation is initiated by thrombin, which converts fibrinogen into fibrin, in a covalent cross-linking reaction mediated by transglutaminase. Thus, for cross-linking on chip, thrombin 8 U/mL (Sigma-Aldrich®, Germany) in EGM-2 was perfused through the vascular (feeding) channels.

3.3 DIFFERENTIATION STAINING

The Alizarin Red S assay is used for the quantification of matrix mineralization; it can bind the calcium cations through a chelation process (Bernar et al., 2022a).

For the Alizarin Red S (Sigma-Aldrich®, Germany) staining, a 2% (w/v) solution was prepared by dissolving 200 mg of powder in 10 mL of distilled water, and after complete dissolution by thoroughly mixing, the solution was filtered with a 0,45 µm filter.

To stain osteoblasts, media was removed, cells were washed with PBS 1X and fixed with 4% paraformaldehyde (PFA) for 15 minutes at room temperature. After fixation, PFA was removed and

cells were washed with distilled water, then 5 mL of Alizarin Red S solution was added to cover the cell layer. After 20 minutes of incubation at room temperature, the staining was removed and cells were washed several times with distilled water to remove the excess dye.

On the other hand, the Alcian Blue dye forms complexes with proteoglycans and glycosaminoglycans highly present in the articular cartilage extracellular matrix (Terry et al., 2000).
A solution of 1% (w/v) Alcian Blue 8GX (Sigma-Aldrich®, Germany) was prepared by dissolving 100 mg of powder in 10 mL of 0.1 M HCl, and after complete dissolution through thoroughly mixing, it was filtered with 0,45 µm filter.
To stain chondrocytes, first the media was removed, then cells were subsequently washed with PBS 1X and fixed with 4% PFA for 15 minutes at room temperature. After removing PFA and washing with PBS, 5 mL of Alcian Blue solution was added to cover the cell layer. After 30 minutes of incubation at room temperature, the staining was removed and cells were washed several times with PBS 1X to remove the excess dye.

3.4 FLS AND HUVECs ENCAPSULATION FOR MEDIA TESTING

FLS were encapsulated in GelMA 5% at 5 x10^6 cells/mL. Five µL drops were deposited in a 48-well plate and subsequently cross-linked by UV light for 1 minute. Different media were then added to evaluate cell viability. Specifically, three media were tested: EGM-2, DMEM/F12 and RPMI.
Similarly, HUVECs were encapsulated in FGT at 6 x 10^6 cells/mL. 5 µL drops of cell-laden hydrogel were deposited in a 48-well plate and subsequently cross-linked with 300 µl of thrombin (8U/ml), for 30 minutes. Then, thrombin was removed and replaced with the same media tested for FLS.
To identify the most suitable culture conditions for the multicellular SoC, different media combinations were subsequently evaluated using HUVEC-laden hydrogels prepared as described above. The following formulations were tested:

- EGM-2 – RPMI 1:1;
- EGM-2 – RPMI 1:4;
- EGM-2 – DMEM/F12 1:1;
- EGM-2 – DMEM/F12 1:4.

In each well, 1mL of medium was added and the cells were cultured for 72h.

3.5 CELLS ENCAPSULATION AND SoC ASSEMBLY

Cells were detached and counted prior to encapsulation, as previously described. Cell suspensions were centrifuged at 1500 rpm for 5 min, and the supernatant was discarded. The resulting cell pellets were resuspended in the appropriate volume of medium to allow dilution of the hydrogel precursor solutions to the desired final concentrations.

Specifically:

- FLS were resuspended at 5 × 10^6 cells/mL in DMEM/F12 and mixed with GelMA and LAP to obtain final concentrations of 5% GelMA and 0.3% LAP;
- Osteoblasts and chondrocytes were resuspended at 5 × 10^6 cells/mL in their respective differentiation media and mixed with GelMA and LAP to obtain final concentrations of 8% GelMA and 0.5% LAP;
- HUVECs were resuspended at 6 × 10^6 cells/mL in EGM-2 and mixed with the FGT hydrogel.

Cell-laden hydrogels were gently mixed to ensure homogeneous cell distribution and subsequently loaded into the SoC. In each channel, 5 µL of cell-laden hydrogel were loaded (**Figure 3A**).

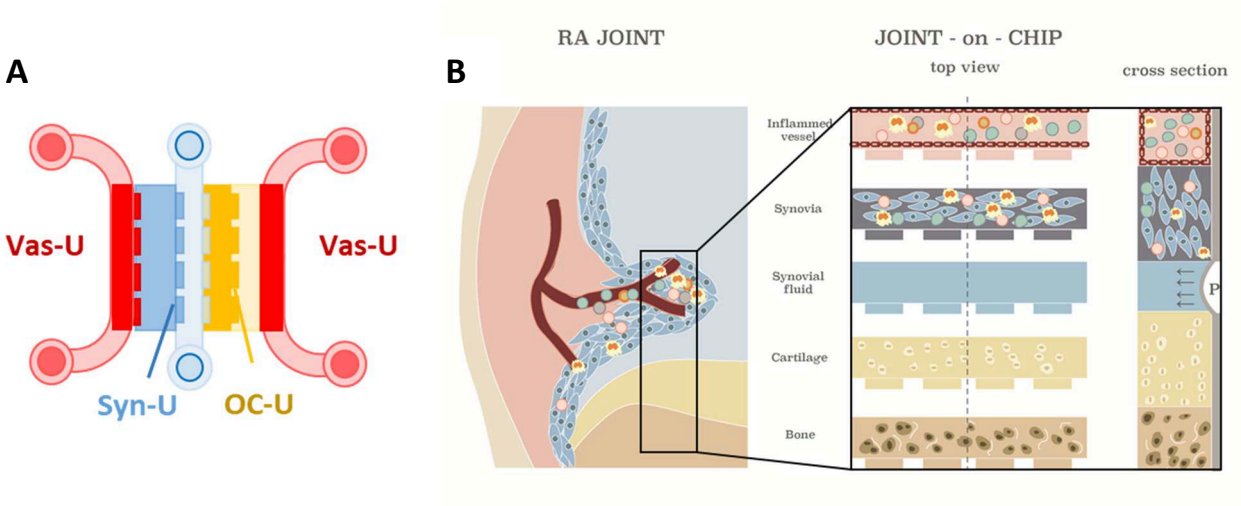

Figure 3*: **SoC platform** (**A**) Schematic representation of the SoC platform, comprising two vascular units (VAS-U), the synovial unit (SYN-U) and the osteo-chondral unit (OC-U). (**B**) Comparison between the cellular organization of the joint and its corresponding reproduction within the multicellular RA SoC, including vascular, synovial, cartilage and bone compartments.*

FLS, chondrocytes and osteoblasts were deposited into their respective channels the chip and exposed under UV light for 1 minute for cross-linking. Then, HUVECs resuspended in FGT were deposited into the vascular channels.

The chips were sealed with a polydimethylsiloxane (PDMS) membrane, closed using screws and bolts, and connected to inlet and outlet tubing. The outlets were then connected to the reservoirs in order to collect the supernatant at different time-points.

To induce enzymatic crosslinking of the FGT hydrogel and promote vessel-like structure formation, thrombin (8 U/mL) was perfused through the HUVECs channels at a flow rate of 6 µl/min for 30 minutes using a microfluidic pump. After 30 minutes of thrombin, the syringes were replaced with the ones with fresh culture medium.

For perfusion, the medium supplied to the FLS compartment consisted of a 1:1 mix of EGM-2 and DMEM/F12, while for the other compartments, a mix of osteogenic and chondrogenic media (1:1) was further diluted 1:1 with EGM-2. Then, the chips were again connected to the perfusion pump at a rate of 6 µL/min for 3 days.

Then, to mimic the inflammatory environment characteristic of the RA-joint, the media were supplemented with pro-inflammatory cytokines: IL-1β (10 ng/mL), IL-6 (10 ng/mL), TNF-α (20 ng/mL) (all from PeproTech, USA). Control chips without cytokines were used to compare the final results. The chips were then perfused until the endpoint of 7 days from the encapsulation (**Figure 4**).

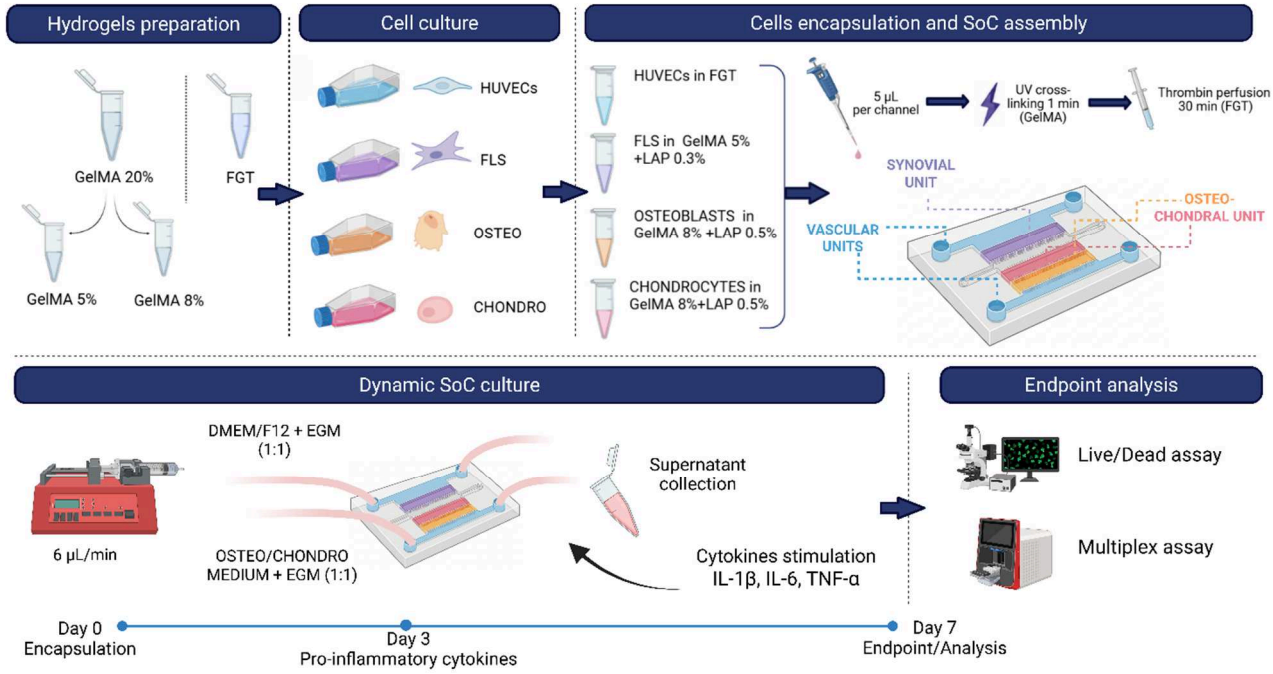

Figure 4*: Experimental workflow for the generation and dynamic culture of the SoC.* *GelMA and FGT hydrogels were prepared and used to encapsulate RA patient-derived FLS differentiated in osteoblasts and chondrocytes, and HUVECs. FLS, osteoblasts and chondrocytes encapsulated in GelMA were loaded into the SoC (5µl per channel). Following UV crosslinking of GelMA, HUVECs encapsulated in FGT were deposited into the vascular units and thrombin was perfused for 30 minutes to induce FGT crosslinking. Subsequently, the synovial compartment was perfused with EGM-2:DMEM/F12 1:1, while the osteochondral compartment was perfused with EGM-2:OSTEO/CHONDRO MEDIUM 1:1*

for 7 days under dynamic flow conditions. On day 3, a cocktail of pro-inflammatory cytokines (IL-1β, IL-6, TNF-α) was added to the SoC to mimic the inflammatory RA microenvironment. At the endpoint, Live/Dead and multiplex assays were performed. Created in Biorender.com.

3.6 VIABILITY STAINING

After 7 days of encapsulation, cell viability within the SoC was assessed using a Live/Dead (L/D) assay. Cells were stained with Calcein-AM (BD Biosciences, USA) which permeates live cells and is converted into fluorescent calcein by intracellular esterases of metabolically active cells and Propidium iodide (PI) (Thermo Fisher Scientific, USA) which selectively penetrates dead cells with disrupted membrane and binds to DNA.

The staining solution consisted of Calcein-AM (2 µM) and PI (50 µg/mL). Prior to staining, supernatants were collected and the chips were washed with PBS 1X. Subsequently, 500 µL of L/D staining solution were perfused into each channel of the SoC and incubated for 20 minutes in the dark. After incubation, the chips were washed with PBS 1X to remove excess reagents and images were acquired using the Thunder Fluorescent Microscope (Leica Biosystems, Germany).

Live cells were identified by green fluorescence, whereas dead cells exhibited red fluorescence.

3.7 MULTIPLEX

Cytokine profiling was performed on chip flow-through using the Bio-Plex Pro™ Human Inflammation Panel 1, 37-Plex (Bio-Rad Laboratories, Hercules, CA, USA). The BioPlex 200 System (Bio-Rad Laboratories, Hercules, CA, USA) allowed the simultaneous analysis of different molecules in a single well. The assay contained dyed beads conjugated with monoclonal antibodies specific for a target protein. Fifty µL of supernatant were used and the concentrations (pg/mL) of the analysed molecules were measured against the standard curve. The analysis was performed according to the manufacturer's instructions. Statistical analysis was performed with GraphPad Prism, version 9.0.0.

4. RESULTS

4.1 Assessment of osteogenic and chondrogenic differentiation

To generate an autologous SoC model capable of reproducing patient-specific pathological features, FLS isolated from RA patients were differentiated using chondrogenic and osteogenic medium to obtain respectively, chondrocytes and osteoblasts. Differentiation was evaluated after 2 and 3 weeks in order to compare the maturation state of the differentiated cells over time. To validate the effectiveness of both osteogenic and chondrogenic differentiation protocols, cells were stained with both Alizarin Red S and Alcian Blue, using undifferentiated FLS as negative control. As shown in **Figure 5 A-B**, FLS were negative for both staining, with no detectable calcium deposition or GAG-enriched ECM formation.

Alizarin Red S staining performed on differentiated osteoblasts revealed evident calcium deposition, stained in red in **Figure 5 D-F**, confirming successful osteogenic differentiation. In particular, after 3 weeks of differentiation, a more intense and homogeneous staining was observed compared to the 2 weeks condition, suggesting increased matrix mineralization and a more mature osteogenic phenotype.

Moreover, Alcian Blue staining performed on differentiated chondrocytes in **Figure 5 C-E**, demonstrated the presence of GAG-rich ECM, in particular in areas where cells were in close proximity to each other, indicative of chondrogenic differentiation.

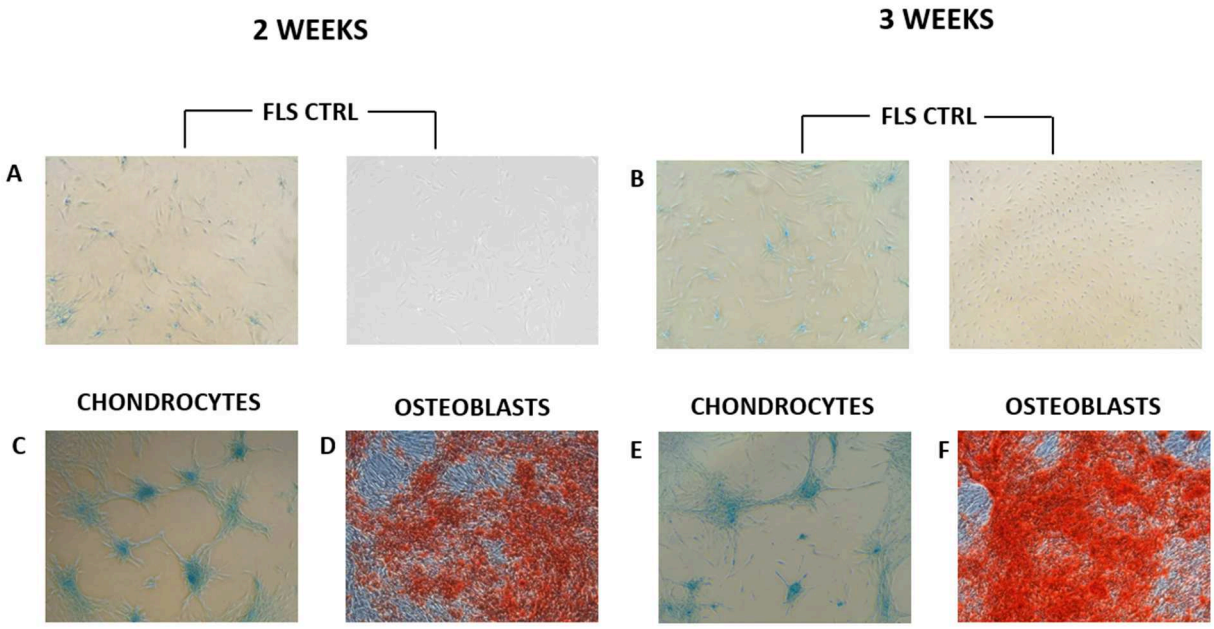

Figure 5*: Osteogenic and chondrogenic differentiation of RA patient-derived FLS after 2 and 3 weeks. Undifferentiated FLS controls were negative for Alcian Blue and Alizarin Red S staining after 2 weeks (**A**) and 3 weeks (**B**). Alcian Blue staining confirmed GAG-rich extracellular matrix production in differentiated chondrocytes after 2 weeks (**C**) and 3 weeks (**E**).Alizarin Red S staining showed progressive calcium deposition in differentiated osteoblasts after 2 weeks (**D**) and 3 weeks (**F**).*

4.2 Optimization of culture media for cells viability and morphology

Since the vascular units of the SoC also function as feeding channels for all cellular compartments, preliminary experiments were performed to identify a culture medium formulation capable of maintaining HUVECs viability and morphology while being compatible with the nutritional requirements of the other cell populations included in the system. To this end, different culture media, including EGM-2, DMEM/F12 and RPMI, were evaluated on HUVECs encapsulated in FGT. As shown in **Figure 6A**, HUVECs viability was highest in EGM-2, whereas a reduction in viable cells was observed in the DMEM/F12 and RPMI conditions.

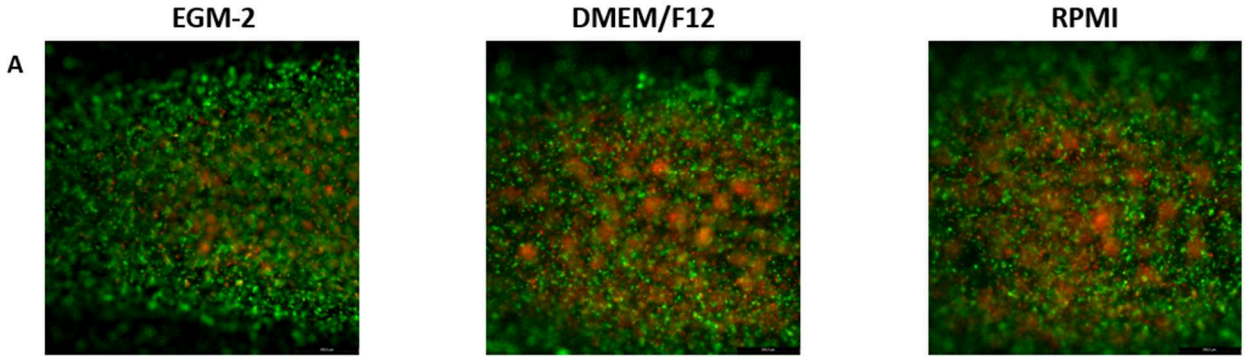

Figure 6*: Media testing on HUVECs encapsulated in FGT.* *72 h after HUVECs encapsulation, cell viability was evaluated by performing Live/Dead, as shown HUVECs remains highly viable when cultured in EGM-2, whereas DMEM/F12 and RPMI conditions showed reduced viability.*

Based on these preliminary observations, different medium combinations containing EGM-2 mixed with either RPMI or DMEM/F12 at different ratios were tested to identify a formulation suitable for both HUVECs and the other cellular populations included in the SoC. This approach was adopted to reduce the proportion of EGM-2, as its high content of growth factors could influence the behaviour of the other cell types, while still maintaining a sufficient proportion of endothelial growth factors, as HUVECs cultured without EGM-2 exhibited reduced viability. Although DMEM/F12 is widely used for FLS, osteoblasts and chondrocytes, the choice of also including RPMI in this experimental setting is to favour future incorporation of immune cells within the system, which are generally cultured in this cell culture medium.

After 72 hours, Live/Dead staining was performed, as shown in **Figure 7**. A comparison between the EGM-2:RPMI 1:1 condition (**A**) and the EGM-2:RPMI 1:4 condition (**B**) indicated higher viability in the first condition. However, despite the viability, HUVECs exhibited a less organized morphology and reduced intercellular connectivity compared to the DMEM-containing conditions.

Indeed, HUVECs cultured in EGM-2:DMEM/F12 mixtures (**Figure 7 C–D**) showed both high viability and improved morphology, characterized by a more homogeneous cell distribution and

enhanced formation of interconnected cellular structures. In particular, the EGM-2:DMEM/F12 1:1 condition (**Figure 7C**) provided the most favourable balance between viability and endothelial-like morphology, whereas increasing the proportion of DMEM/F12 to 80% seemed to result in reduced cell viability.

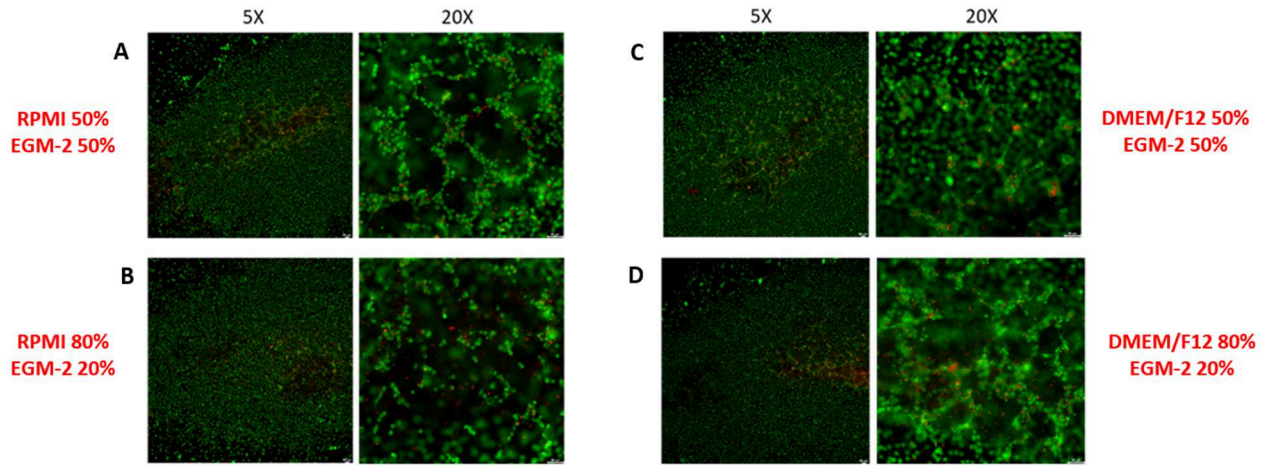

Figure 7*: **Evaluation of different media combinations for HUVECs culture.** HUVECs were encapsulated in FGT and cultured for 72h using different culture medium combinations to identify the most suitable condition for preserving endothelial cell viability and morphology while supporting the nutritional requirements of the other cells populations within the SoC. HUVECs were cultured in different mixtures of EGM-2 with RPMI or DMEM/F12 at varying ratios: (**A**) EGM-2:RPMI 1:1, (**B**) EGM-2:RPMI 1:4, (**C**) EGM-2 DMEM/*F12 1:1 and (**D**) EGM-2:DMEM/*F12 1:4. Representative images at 5X and 20X magnification show the Live/Dead staining (**Calcein AM/Propidium Iodide**) performed after 72 hours, in which green fluorescence indicates live cells while red fluorescence indicates dead cells. Condition (**C**) showed the best balance between HUVECs viability and endothelial-like morphology.*

Although the EGM-2:DMEM/F12 1:1 formulation emerged as the most promising condition for HUVECs, the SoC is designed to host multiple cell populations with distinct nutritional requirements. Therefore, additional experiments were performed to evaluate whether the tested culture media could also support the viability of FLS encapsulated in GelMA 5%.

As shown in **Figure 8A**, FLS maintained high viability after 72h of culture in all tested conditions, including EGM-2, DMEM/F12 and RPMI.

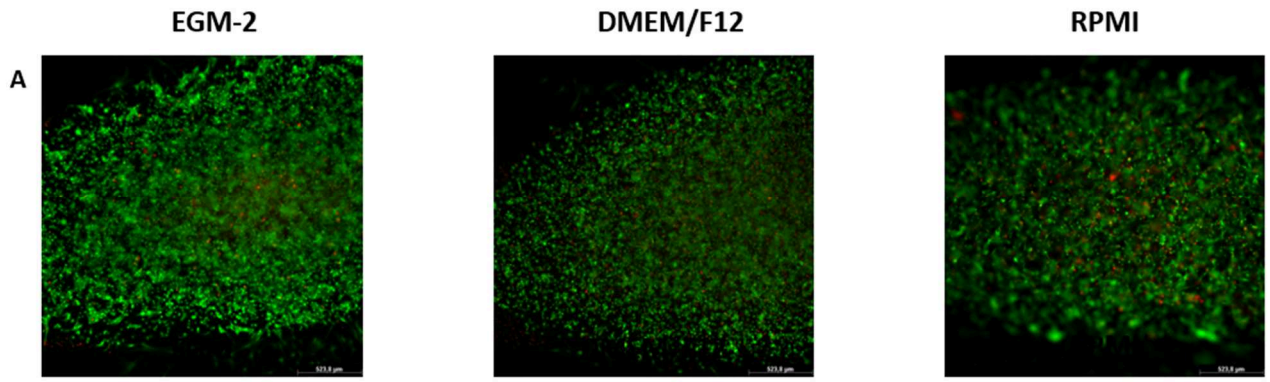

Figure 8: Media testing on FLS encapsulated in GelMA 5%. *72 h after FLS encapsulation, cell viability was evaluated by performing Live/Dead, as shown in the figure FLS remain viable in all the three conditions, without significant differences.*

Overall, the condition with the same proportion of DMEM/F12 and EGM-2 (**Figure 7C**) was selected as the most suitable to be used in the SoC to maintain HUVECs viability and morphology while ensuring suitable culture conditions for the other cellular compartments.

4.3 Viability and morphology evaluation of SoC under spontaneous and inflamed conditions

The multicellular SoC was generated by encapsulating patient-derived FLS, differentiated chondrocytes and osteoblasts, and HUVECs within the different compartments of the device in order to reproduce key cellular features of the RA joint microenvironment. The experiments were conducted under two different settings: under spontaneous conditions (No Cyto), without the addition of external stimuli (**Figure 9A**), and exposed to pro-inflammatory stimuli (Cyto) three days after encapsulation through the administration of a cytokines cocktail containing IL-1β, IL-6 and TNF-α to mimic the RA-like inflammatory conditions (**Figure 9B**).

After 1 week of culture, Live/Dead staining was performed to evaluate cell viability and morphology within the different cellular compartments of the SoC. Comparison between the two conditions did not reveal differences in overall cell viability, as it was maintained high across all compartments in both spontaneous and inflamed conditions.

Regarding cell morphology, inflammatory stimulation appeared to induce morphological changes in several cellular populations. FLS (**SYN-U**) exhibited a more elongated morphology and formed more evident interconnected cellular structures under cytokine stimulation (**Figure 9B**). Chondrocytes (**Chondro**) appeared more elongated and spatially organized, while osteoblasts (**Osteo**) showed a more spread and interconnected morphology compared to the spontaneous condition. Regarding the

vascular compartments (**VAS-U**), HUVECs displayed a similar morphology in both No cyto and Cyto conditions (**Figure 9A-B**) with no significant differences observed.

These observations suggest that the exposure to pro-inflammatory cytokines promoted morphological changes and structural reorganization without compromising overall viability of the SoC system, which is also maintained under spontaneous condition.

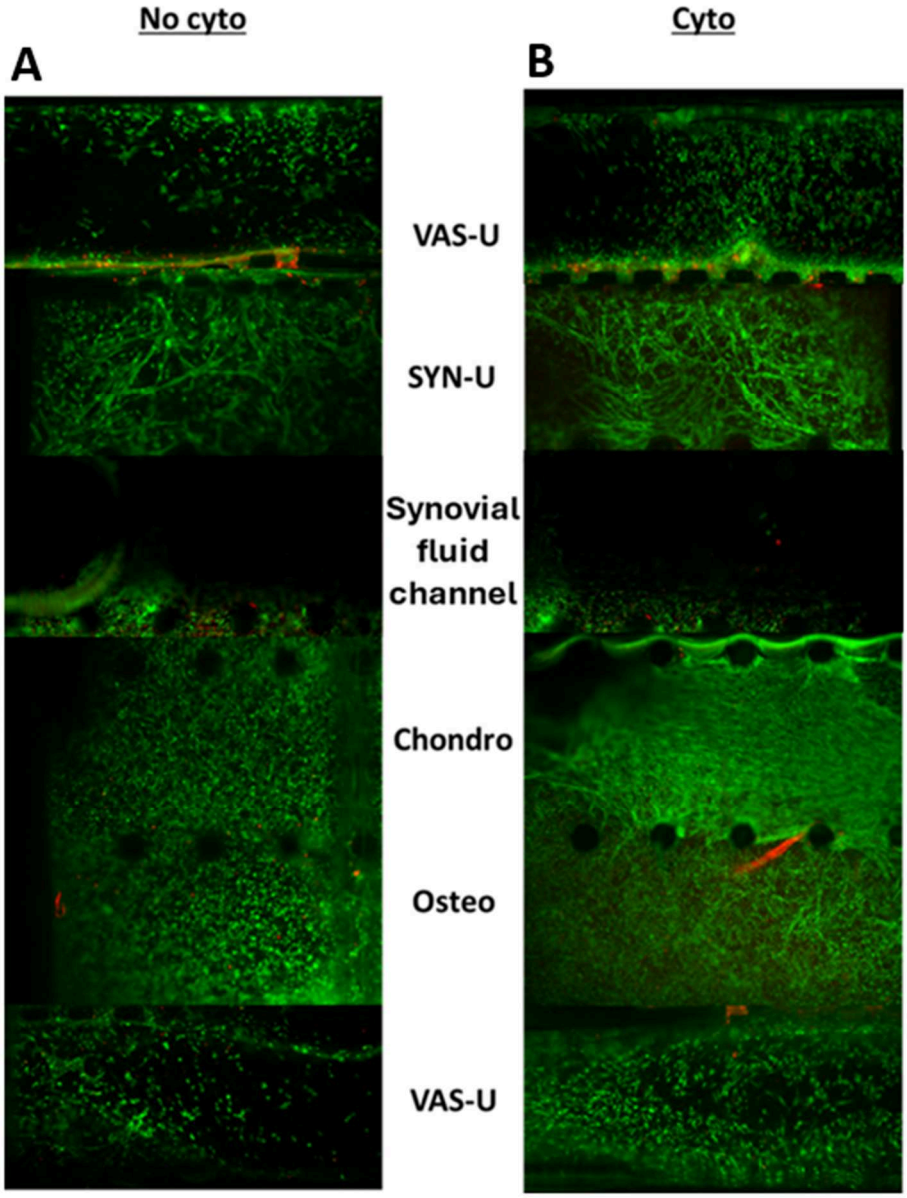

Figure 9*: Live/Dead assessment of the SoC under spontaneous and inflammatory conditions. Cells were encapsulated within the SoC and cultured under spontaneous conditions, without cytokine stimulation (**A, No cyto**) or with a cocktail of pro-inflammatory cytokines containing IL-1β, IL-6, TNF-α (**B, Cyto**). Live/Dead staining (**Calcein AM/Propidium iodide**) was performed after 1 week of culture to evaluate cell viability and morphology within the different cellular compartments of the SoC. Overall, the viability of the cells is preserved in both conditions (**A-B**), while regarding the*

*morphology we can appreciate differences in several cellular compartments between the two conditions. Specifically, FLS exhibited a more elongated and interconnected cellular network under inflammatory conditions (**SYN-U B**), while chondrocytes and osteoblasts also displayed a more elongated and spatially organized morphology following exposure to pro-inflammatory cytokines (**Osteo-Chondro B**).*

4.4 Cytokines analysis of the SoC supernatants after 1 week of culture

After 1 week of culture, supernatants from the SoC were collected and analysed using a 37-plex multiplex cytokine assay to evaluate the response of the model to pro-inflammatory stimulation. Cytokine levels were compared between spontaneous (No Cyto) and inflammatory (Cyto) conditions. Among the 37 analysed cytokines, 33 showed significantly higher levels under inflammatory conditions compared to spontaneous conditions, while 4 (Chitinase-3-like 1, Osteopontin, Pentraxin-3, TNF-R1) were not significantly different (**Figure 10**).

The increased secretion of the majority of the analysed cytokines in the inflamed SoC demonstrates that the model is biologically responsive to pro-inflammatory stimuli.

These results support the potential use of SoC platform as an *in vitro* RA model for studying disease mechanisms and for testing the efficacy of anti-inflammatory and disease-modifying drugs.

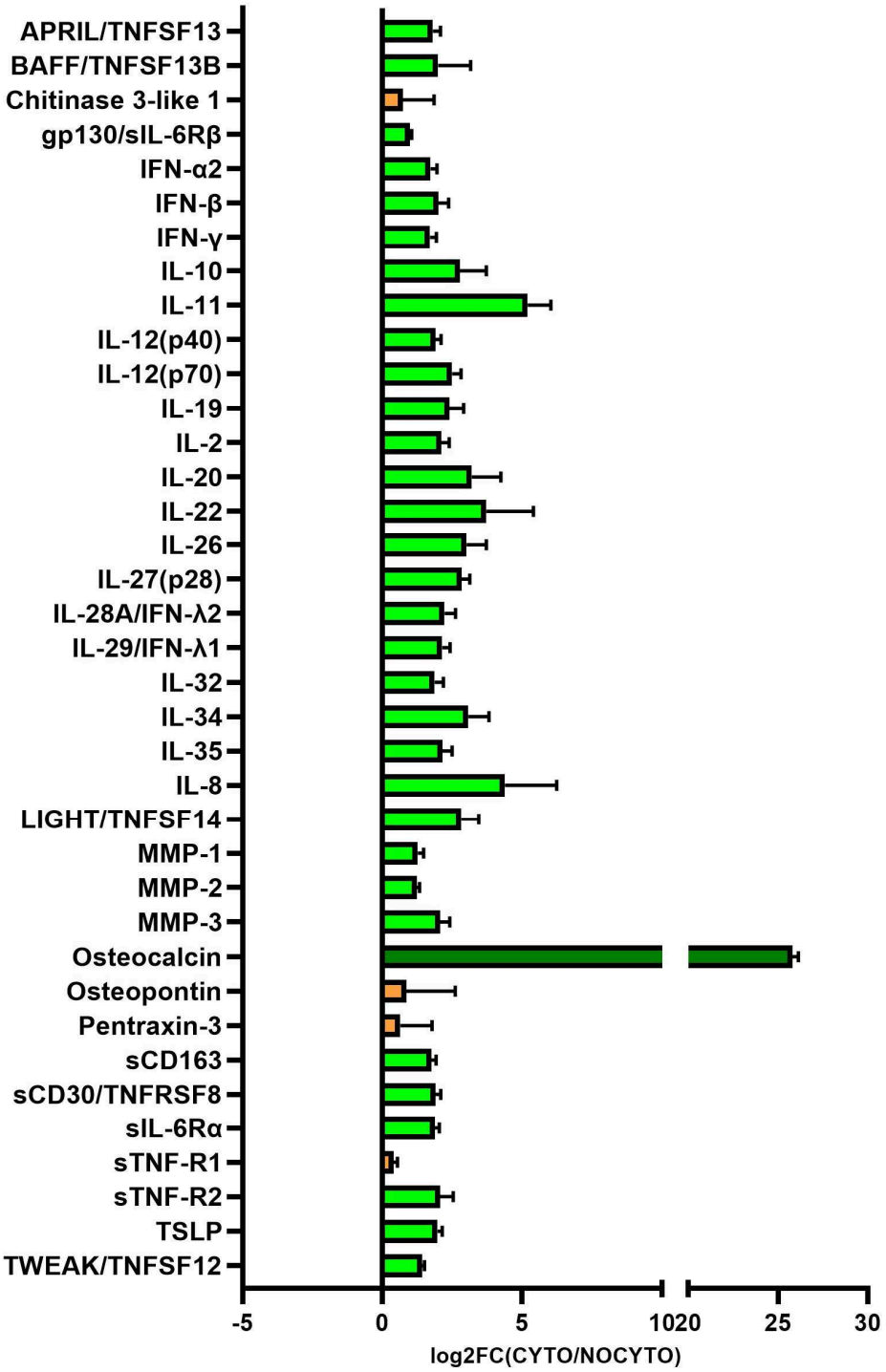

Figure 10*: Cytokine profiling of the SoC supernatants under spontaneous and inflammatory conditions. A 37-plex multiplex cytokine assay was performed on supernatants collected from the SoC after 1 week of culture. Cytokines levels were compared between spontaneous condition (NO CYTO) and pro-inflammatory condition (CYTO and represented as log2 fold change (CYTO/NOCYTO). Data are represented as mean ± SD (n=3) and statistical analysis was performed using paired t-test. Statistically significant differences are represented as green bars, while not significant are represented as orange bars.*

5. DISCUSSION

RA is a chronic systemic autoimmune disease characterized by persistent synovial inflammation and progressive joint destruction, ultimately leading to deformity, functional impairment and reduced quality of life. Although the etiology of RA is still not completely understood, its multifactorial origin, involving both genetic and environmental factors, is widely recognized (Gao et al., 2024). The interplay between these factors leads to the loss of immune tolerance, production of autoantibodies, and constant recruitment and activation of immune cells, leading to the secretion of pro-inflammatory cytokines that sustain chronic inflammation and progressive joint damage (van Delft & Huizinga, 2020). In addition to immune cells, resident joints cells play a crucial role in RA pathogenesis, as activated osteoclasts promote bone erosion, while invasive FLS contribute to cartilage degradation and perpetuation of the inflammatory microenvironment (Noss & Brenner, 2008).

Despite the increasing number of therapeutic options available for RA, a substantial proportion of patients fail to achieve an adequate clinical response, which may manifest as non-response, limited efficacy over time or intolerance to therapy. This variability is largely due to the heterogeneity of RA, as patients exhibit distinct disease phenotypes, progression rates and therapeutic responses (Huang et al., 2021; Mueller et al., 2021).

Currently, reliable biomarkers capable of predicting therapeutic response and guiding treatment selection are still lacking and therapeutic decisions still largely rely on a trial-and error approach. Consequently, patients may be unnecessarily exposed to ineffective or potentially toxic treatment, delaying the achievement of disease control and increasing healthcare costs, thereby contributing to the substantial socioeconomic burden associated with RA (Gudu et al., 2025).

This scenario highlights the urgent need of personalized medicine approaches in RA, as well as for advanced experimental models capable of accurately recapitulating the complexity of the rheumatoid joint microenvironment and its patient-specific features.

In this context, the European project FLAMIN-GO was established with the aim of developing a patient-specific SoC platform capable of reproducing the complex cellular and molecular interactions occurring within the RA joint. By integrating patient-derived tissues into a microfluidic system and generating an autologous SoC, the project seeks to provide a physiologically relevant model for investigating RA pathogenesis, identifying novel therapeutic strategies and supporting precision medicine approaches through patient-specific drug testing and treatment selection.

Within this framework, this thesis focused on the assembly and biological validation of a multicellular RA-SoC model comprising synovial, osteochondral and vascular compartments. Specifically, the aim was to evaluate the viability, morphology and biological responsiveness of the platform under both spontaneous and inflammatory conditions. The establishment of this model represents the foundation

for the development of a fully competent SoC, enabling the future integration of the immune system and the assessment of therapeutic efficacy in a patient-specific setting.

To generate patient-specific multicellular RA-SoC model, FLS derived from RA patient biopsies were differentiated into chondrocytes and osteoblasts. This approach was supported by previous studies demonstrating that FLS possess mesenchymal-like characteristics and exhibit multilineage differentiation potential, including the ability to differentiate into osteoblasts, chondrocytes and also adipocytes (Ding et al., 2022; Liu et al., 2010). Osteogenic differentiation was assessed by Alizarin Red S staining, which detects the calcium deposits within the ECM and is widely used as a marker of matrix mineralization and osteoblast maturation (Bernar et al., 2022b). The positive staining observed in differentiated osteoblasts cultures confirmed the successful acquisition of an osteogenic phenotype. These results are consistent with those reported by (Ding et al., 2022) who demonstrated successful osteogenic differentiation of FLS through positive Alizarin Red S staining and increased calcium deposition following prolonged culture under osteogenic conditions. Similarly, chondrogenic differentiation was assessed by Alcian Blue, which stains GAGs and proteoglycans, major components of the cartilage ECM. The positive staining observed in differentiated culture indicates the synthesis of cartilage-like matrix and confirms the successful chondrogenic differentiation of FLS. Similar results were reported by (Pelassa et al., 2025), which showed that FLS exposed to chondrogenic stimuli acquire a chondrocyte-like phenotype and produce GAG-rich ECM characteristic of cartilage tissue.
Overall, the successful differentiation into osteoblast and chondrocytes from patient-derived FLS enabled the establishment of an autologous osteochondral compartment, representing a crucial step towards the development of a patient-specific RA-SoC model.

Another key aspect in the assembly of the SoC was the establishment of a functional vascular compartment. In our platform, the vascular units were designed not only to reproduce the endothelial component of the joint microenvironment, but also to serve as feeding channels, ensuring the delivery of nutrients to the other tissue compartments.
Although the synovial and osteochondral compartments were entirely generated from patient-derived cells, the vascular compartment was established using commercially available HUVECs, as the generation of functional endothelial cells from synovial biopsy-derived FLS remains challenging and has not yet provided a reliable differentiation strategy. Consequently, the current version of the platform cannot yet be considered fully autologous. However, this limitation is shared by many vascularized OoC systems (Diao et al., 2025; Quintard et al., 2024; Thompson et al., 2023), where HUVECs remain the most widely used endothelial cell source because of their availability and ease

of use. Furthermore, the generation of patient-specific endothelial cells from peripheral blood remains technically challenging due to the low abundance of endothelial progenitor populations and the complexity of isolation and differentiation procedures (Ormiston et al., 2015). Therefore, HUVECs represented a practical and reliable choice for the development and validation of the vascular compartment.

To support the coexistence of all cellular populations within the platform, preliminary experiments were performed to identify a culture medium capable of preserving endothelial viability and morphology while remaining compatible with the metabolic requirements of the other cellular compartments. Indeed, EGM-2 contains many growth factors and potential pro-inflammatory molecules such as vascular endothelial growth factor (VEGF) (Reinders et al., 2003), which raise the need to reduce to a minimum the use of this cell culture medium within the SoC in order to create a physiological system. Although HUVEC viability remained relatively high across all conditions, morphological differences emerged when comparing the use of EGM-2 in combination with DMEM/F12 instead of EGM-2 with RPMI. In particular, EGM-2:DMEM/F12 1:1 mixture promoted a more homogenous cell distribution and enhanced intercellular connectivity. Since endothelial morphology and cell-cell interactions are associated with vascular network formation and functionality (Tsuji-Tamura & Ogawa, 2018), these observations suggest that this condition provided the most suitable microenvironment for maintaining endothelial characteristics. The improved morphology observed under this condition is consistent with findings with previous vascularized organoid-on-chip studies, demonstrating that balanced culture media are often required to preserve endothelial self-organization and vascular network formation while supporting the metabolic requirements of multiple cell types within the same OoC platform (Quintard et al., 2024). Therefore, EGM-2:DMEM/F12 1:1 formulation was selected for subsequent SoC experiments, representing the most suitable compromise between the specific requirements of HUVECs, the need to support viability of all cellular compartments included in the platform and minimizing the potential pro-inflammatory effects of bioactive components present in EGM-2 medium.

Following these preliminary optimization studies, the complete multicellular RA-SoC was assembled and cell viability and morphology were evaluated under both spontaneous and pro-inflammatory conditions, mimicking key features of the RA joint microenvironment.

Overall, high cell viability was maintained across all compartments under both spontaneous and inflammatory conditions, demonstrating the ability of the platform to support the co-culture of multiple cell types within a microfluidic environment. Beyond cell survival, the preservation of cellular morphology and the coexistence of different tissue compartments are essential features of

physiologically relevant OoC systems, as they indicate the maintenance of tissue-specific functions and biologically meaningful cell behaviour (Low et al., 2021).

As the pro-inflammatory stimulation was intended to mimic the chronic inflammatory environment of the RA joint, the preservation of cell viability constitutes an important prerequisite for the biological relevance of the model. Indeed, the cytokine cocktail induced a biological response without causing cytotoxic effects, indicating that the model remained functional while responding to inflammatory cues. This suggests that the selected inflammatory conditions were sufficient to trigger cellular activation while preserving the overall integrity of the multicellular system.

In addition to maintaining viability, inflammatory stimulation induced morphological changes in several cellular compartments of the SoC. In particular, we observed that FLS displayed a more elongated morphology and formed more evident interconnected cellular structures compared with the spontaneous conditions. These observations are supported by the well-established role of pro-inflammatory cytokines, such as TNF-α and IL-1β, in promoting FLS activation and the acquisition of an aggressive phenotype characterized by enhanced migration, invasiveness and tissue-remodelling capacity (Bartok & Firestein, 2010; Noss & Brenner, 2008).

Moreover, previous studies have demonstrated that inflammatory cytokines can directly affect FLS morphology and cytoskeletal organization. In particular, Filali and colleagues showed that exposure of RA-FLS to pro-inflammatory cytokines, such as TNF-α and IL-17, induced cell elongation, increased pseudopodia formation and actin cytoskeleton remodelling reflecting the acquisition of an activated phenotype and enhanced interactions with surrounding cells (Filali et al., 2023). Therefore, the morphological changes observed in our inflamed SoC further support the ability of the cytokine stimulation to reproduce key pathological features of the RA synovial microenvironment.

Interestingly, we also observed morphological changes in chondrocytes and osteoblasts, which appeared more elongated and spatially organized following cytokine stimulation. In particular, these observations are consistent with previous studies demonstrating that exposure of chondrocytes to pro-inflammatory cytokines, such as IL-1β or TNF-α, induces a transition of the morphology toward a more elongated fibroblast-like morphology, along with cytoskeletal reorganization, increased F-actin organization and stress fibre formation (Chen et al., 2015). Regarding osteoblasts, we observed a more spread and interconnected morphology under pro-inflammatory conditions. These observations may reflect an active cellular response to the inflammatory microenvironment. Indeed, Bousch et al reported that exposure of osteoblasts to pro-inflammatory cytokines, including TNF-α and IL-1β, enhanced their proliferation, metabolic activity and mineralization capacity (Bousch et al., 2024). Although osteogenic activity was not directly assessed in our model following cytokine stimulation, the observed morphological reorganization suggests that osteoblasts remained responsive to

inflammatory cues and may have undergone functional adaptations associated with bone remodelling processes.

These observations suggest that the inflammatory stimulation elicited biologically relevant responses not only in the synovial compartment but also within the osteochondral tissues. The morphological changes observed across multiple cellular compartments further support the ability of the cytokine stimulation to reproduce key features of the inflammatory joint microenvironment.

Overall, these findings indicate that the multicellular RA-SoC remained viable while responding to inflammatory cues through cell-type-specific morphological adaptations. This is particularly relevant because RA is characterized by dynamic interactions between synovial, cartilage, bone and vascular compartments rather than by isolated cellular responses (Ganeshalingam et al., 2025). Therefore, the ability of the platform to sustain multiple cell populations and reproduce early inflammation-associated phenotypic changes supports its potential as a physiologically relevant model of the rheumatoid joint.

The final objective of this thesis was to assess whether our RA-SoC platform, beyond maintaining cell viability and displaying inflammation-induced morphological changes, was also capable to biologically respond to pro-inflammatory stimulation. Demonstrating such functionality is essential to validate the physiological relevance of the model and its ability to reproduce key features of the RA joint microenvironment. To this end, a 37-plex cytokine assay was performed to compare the secretory profiles under the spontaneous and inflamed conditions.

The analysis revealed a clear distinction between the two conditions, with the inflamed SoC characterized by the increased secretion of several pro-inflammatory mediators. These findings indicate that the cytokine stimulation was effective in activating the multicellular SoC and triggering inflammatory pathways associated with RA pathogenesis (Fujimoto & Niiro, 2025).

Among the 33 cytokines significantly upregulated under inflammatory conditions, we identified several mediators involved in bone remodelling and joint destruction, including IL-34, IL-20 and TNF-β. The detection of IL-34 is particularly interestingly, as previous studies have demonstrated that TNF-α and IL-1β stimulation induces IL-34 production by RA-FLS (Chemel et al., 2012). Given the established role of IL-34 in pathological bone erosion, its increased secretion suggests that the activated synovial compartment of the SoC is capable of generating signals involved in the bone-destructive processes characteristic of RA. Similarly, we found elevated levels of IL-20 under inflammatory conditions. IL-20 has been implicated in bone remodelling and has been associated with the regulation of osteoclastogenesis, as demonstrated by (Meng et al., 2020). They showed that IL-20 acts together with RANKL/OPG axis in upstream regulation of osteoclastogenesis and bone

erosion. Together, the upregulation of IL-34 and IL-20 supports the activation of molecular pathways associated with inflammatory bone destruction.

In addition, we found increased levels of MMP-1 and MMP-2, indicating the activation of tissue-remodelling mechanisms. Specifically, previous studies reported that rheumatoid synovial cells stimulated with IL-1β are induced to secrete pro-MMP2 and its active form, which is responsible for collagen fibres degradation (Honda et al., 2008). Interestingly another study demonstrated that the stimulation of chondrocytes with TNF-α enhanced the expression of membrane- type 1 MPP (MT1-MMP), a key activator of pro-MMP2 (Imai et al., 1997). Based on findings, we can speculate that molecular interactions within the multicellular microenvironment may recapitulate mechanisms occurring in the RA joint.

Furthermore, we detected elevated levels of LIGHT (TNFSF14) and IL-32 in the inflamed condition. Our findings are consistent with previous studies demonstrating that TNF-α stimulation of RA-FLS enhanced the release of IL-32, which is highly expressed in RA-FLS, where it contributes to the amplification of the inflammatory signalling (Alsaleh et al., 2010). Similarly, LIGHT has been found to be highly expressed in RA synovium and to promote the production of pro-inflammatory mediators, while also contributing to FLS proliferation and activation (Ishida et al., 2008).

In conclusion, the cytokine profile generated by the inflamed RA-SoC reflected several hallmarks processes of RA pathology, demonstrating that our model is biologically responsive to pro-inflammatory stimuli and capable of reproducing key aspects of the complex multicellular microenvironment of the RA joint. Together with the preservation of cell viability, these findings support the physiological relevance of the RA-SoC and highlight its potential as a patient-specific model for studying RA pathogenesis and evaluating therapeutic responses.

This autologous RA-SoC model has the potential to be exploited for preclinical drug testing, supporting the selection of the most appropriate therapeutic strategy for individual patients. In future developments, we aim to incorporate the patient-derived immune cells, representing a further step towards a fully autologous system capable of recapitulating patient-specific disease characteristics more comprehensively. In addition, the integration of a mechanical actuator would allow the application of controlled pressure to better mimic synovial mechanical stress. Overall, this platform may serve as a valuable tool to investigate molecular mechanisms of RA, as well as to identify novel therapeutic targets and potential biomarkers for diagnosis and prognosis.

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