Performance of nasal chondrocyte-based engineered tissues in osteoarthritis simulating environments

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"The Universe is made of stories,
not of atoms."

Muriel Rukeyser

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1 Summary

Osteoarthritis (OA) is the most prevalent musculoskeletal disease in humans, characterized by a progressive degeneration of the articulation that causes pain and disability in a large percentage of the population. In this pathology, the joint environment becomes predominantly catabolic and concentrations of circulating pro-inflammatory factors significantly increase as compared to non-pathological conditions. Up to date, existing treatments can be effective in reduction of pain and improvement of mobility, but none of the available therapies is able to stop the progression of the disease.

Non-degenerative cartilaginous lesions can be currently treated with cell-based approaches, consisting of the implantation of autologous chondrocytes into the defect site, those cells being isolated from presumable non-affected areas of articular cartilage. Differentially, OA is considered a contraindication for such treatments and, in the scarce cases they have been used for patients with degenerative traces, failure is reported as the more common long-term outcome. Possible causes of these results are the inferior chondrogenic capacity and phenotype stability demonstrated for articular chondrocytes (AC) harvested from affected joints, but also, the detrimental conditions of the OA environment, potentially compromising the performance of any implanted cell-based product.

Nasal chondrocytes (NC) represent an alternative source for cell and tissue engineering approaches, since they can be obtained from a compartment that is not affected (i.e., the nasal septum), and show more reproducible capacity to generate functional cartilaginous tissues as well as similar responses to mechanical and inflammatory stimuli than AC. In fact, tissue engineered cartilage derived from nasal chondrocytes (N-TEC) have been already used in the clinic for the treatment of post-traumatic cartilage lesions, but not results are generated regarding their potential to additionally treat OA defects. In order to assess such potential, it is necessary to evaluate if N-TEC

can survive and maintain their tissue-like properties in the pro-inflammatory and catabolic OA environment, to which cells from the different joint tissues (cartilage, synovial membrane and subchondral bone) contribute.

The pillar of this thesis, my PhD dissertation, consists on the exploration of the suitability of N-TEC for the treatment of OA lesions. Therefore, this manuscript summarizes methods and outcomes resulting from investigating the interactions between nasal chondrocytes and cells/tissues from OA-joints, as an approach to establish the possible compatibility of N-TEC within an OA cartilage defect. Results showed that N-TEC could maintain their cartilaginous properties, when exposed in vitro to inflammatory stimuli as those found in OA joints, and positively influence the inflammatory profile of cells from OA joints through secreted factors. Moreover, N-TEC were able to survive and engraft into OA compartments simulated in vivo, while preserving cartilaginous matrix properties and dampening inflammation, as observed in vitro.

Acknowledging the positive and wide compatibility of N-TEC within OA environments that I demonstrated, the clinical application of autologous N-TEC was tested in two patients with advanced OA, who would have been otherwise considered for partial knee – prosthetic - replacement. After 14 months of implantation, patients have reported reduced pain as well as improved joint function and life quality; all findings indicating that N-TEC can be envisioned as a therapeutic approach for the repair of osteoarthritic knee cartilage defects. To assess efficacy of this procedure, a phase II trial would be required in a larger cohort of patients.

2 Aims of the thesis

2.1 General aim

Investigate the compatibility of tissue engineered cartilage generated with nasal chondrocytes (N-TEC) with an osteoarthritic (OA) simulated environment.

2.2 Specific aims

I. Determine the capacity of N-TEC to form and maintain cartilaginous properties, when exposed in vitro to factors secreted by OA cells/tissues and pro-inflammatory cytokines.

II. Evaluate the potency of N-TEC released factors to damper the production of inflammatory and catabolic molecules in OA cells/tissues in vitro.

III. Assess the capability of N-TEC to survive, maintain the cartilaginous matrix and integrate with surrounding tissues in OA-environments simulated in vivo.

3 State of the art

Being the goal of this thesis to assess the potential of a tissue engineering approach for the treatment of a specific type of cartilage disease, this state of the art chapter presents the principles of the tissue engineering field and how they have been explored for different cartilage repair approaches, in which nasal chondrocytes have emerged as a promising cell source. Specific characteristics of the disease as well as some suitable models for investigating it, are additionally described in the final part of this chapter.

3.1 Tissue engineering (TE)

Since ancient times manhood have been attracted by the idea of creating living entities, and this imaginary has taken us to conceive units with which to replace, repair or regenerate body portions of complex whole organisms *(1)*. Popular literary and artistic pieces as the Greek myth of eternally devoured – and therefore regenerated - liver of Prometheus *(2)* or the more modern and well known "Lab-created" Frankenstein *(3)*, well document this curiosity.

For either the achievement of merely structural reposition, a gain in functionality or a combination of both, the possibilities to succeed in this intention have evolved along with the scientific and technological progresses. While first reports of used substitutes refer to glass eyes as well as extremities in wood or metal *(4, 5)*, nowadays prosthesis range between solely material-based implants *(6)* and grafts of biological origin *(7)*.

Currently, focus is set in the definition of an optimal combination of both materialistic and biological approaches that can match the requirements of mechanical stability and biological compatibility for different applications *(8–10)*. In this search, the possibility of obtaining better biomimetic manners and replacing more specialized structures like tissues and organs - whose availability is naturally limited -, came to live with the emergence of the tissue engineering concept

(11). The paradigm of this field aims for building up bio-equivalent constructs, by combining cell supports (scaffolds) with biological and chemical cues such as cells and differentiating/proliferating factors, with the final goal of targeting clinical applications (Fig. 1).

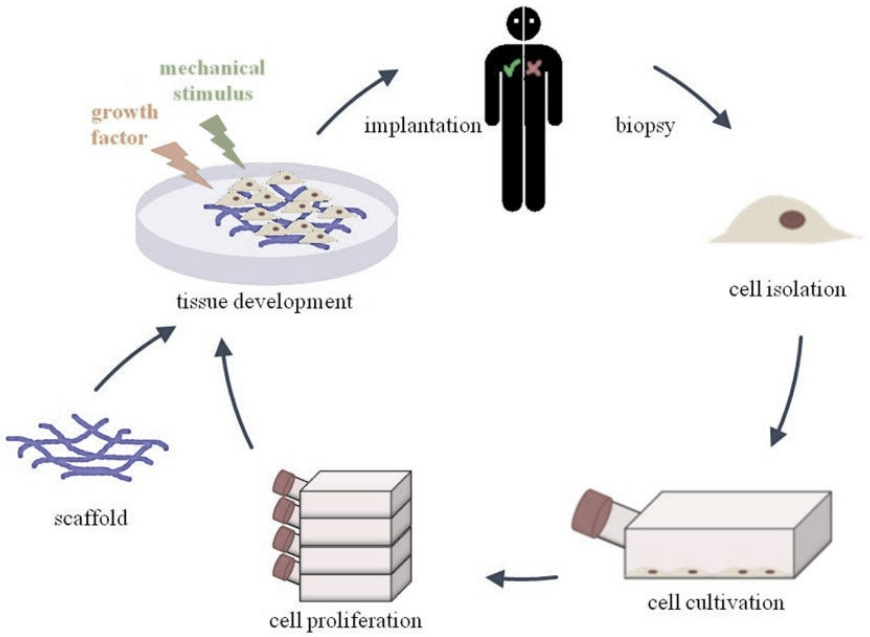

Figure 1. Principles of the tissue engineering (TE) paradigm. Cells isolated from donor biopsies/tissues are cultured in vitro and induced to proliferate and/or differentiate in cell supporting materials such as culture vessels or scaffolds. Chemical and biological cues (factors) are additionally used to promote the formation of targeted three-dimensional biological equivalent. Developed structures are then implanted into the patient in an autologous manner (original image from (12)).

3.1.1 Scaffolds

As one of the primary components of the TE paradigm, the optimal determination of used scaffolds is critical. As a pre-requisite, explored scaffolds must be biocompatible (i.e., non-toxic, non-hazardous, physiologically inert and non-inducers of immunological rejection), besides of supporting – or even better promoting- cellular processes of adherence, proliferation and differentiation *(11)*.

Synthetic materials like metal, ceramics and polymers well fulfill this requirements, and have been exploited for applications in hard tissues *(13)* as the current standard choices in dentistry and orthopedic practices. That is the case for example, of the usage of titanium and hydroxyapatite in - and as - bone substitutes *(14–16)*. However, such materials are not suitable when biodegradability or malleability is desired, as in the case of soft tissues. In those applications, hydrogels and meshes are preferred instead, such as in the generation of skin or vascular substitutes *(17–19)*.

In both cases, development of fabrication technologies has enabled the customization of diverse scaffold features like stiffness and porosity in terms of mechanical properties, or adherence and degradation kinetics from the biological/chemical perspective *(20)*. Specifications in composition for more precise applications are also an alternative, by means of coating (or decoration) with molecules of interest as well as the production based on ECM components from the desired environment. Of this kind, is the selected scaffolding material for some of the experimental setups in this thesis. Those materials could be produced by either de novo formation or de-cellularization of a prefabricated matrix *(21)*. Further possibilities are offered by the developed three dimensional printing techniques, allowing not only the functionalization of scaffolds - with the modelling composition and structure -, but also the design of anatomically shaped cell supports *(22, 23)*. Fine-tuning of all mentioned features for specific applications is the focus of current research, and the evolution of this field dependent on the biological requirements of employed factors and cells, as described below.

3.1.2 Bioactive factors

Aiming for the formation of intended structures with stable and defined biological functions, the identification of critical cues triggering different levels of cell/tissue specification has been another big focus on the field of tissue engineering.

Starting from a basic mix of components supporting the more broad metabolic cellular needs of diverse cell types, the formulation of basal media containing salts, buffering solutions and basic nutrients like amino acids and glucose, served as preliminary means for maintaining cell viability *(24)*. Definition of particular cocktails to sustain more specialized cell types and promote proliferation and differentiation, involved the addition of complementary molecules including hormones and growth factors. Thus can be linked either with the niche of the cells and expected biological function, or generic for diverse phenotypes as is the supplementation with blood serum *(25)*.

Specifically concerning stimulation of cell differentiation, one commonly employed strategy is the inclusion of ECM components in the used scaffolds *(21, 26, 27)*. Regarding soluble factors, the elucidation of cell-specific differentiation pathways is fundamental in order to provide appropriate stimulation. Among the more commonly used, bone morphogenic proteins (BMP) as wells as the transforming, epidermal, fibroblastic, platelet derived and vascular endothelial growth factors (TGF-α/β, EGF, FGF-2, PDGF-AA/AB/BB and VEGF), are shown to contribute not only with lineage specification, but also with survival and proliferation of an extensive panel of cell phenotypes *(28)*, as the ones presented in the following section. A broader description of the function of these bioactive factors is presented in the *section 3.2.7.2.*

3.1.3 Cells

Regarding these critical elements of the TE paradigm, the potential of different sources such as animal, fetal or patient tissues was initially contemplated for cell procurement *(11)*. Later, the discovery and popularity of *'stem cells'*, canonically defined by their abilities of self-renewal and differentiation into mature specialized cells, attracted the interest of the scientific community *(29,*

30), guiding tissue engineering efforts towards their exploitation as cell source in view of regenerative medicine applications *(31)*.

These stem cell populations are categorized according to their niche as embryonic (ESC) or adult stem/progenitor cells (ASC) if isolated from tissues after birth; but also, in consideration of their potency for giving rise to cell phenotypes from all lineages (totipotent), lineages derived from the three embryonic layers (pluripotent) or, from one specific lineage (multipotent) *(32, 33)*. The diversity of origins and features of these cells conveys a wide range of advantages and limitations. ESC for example *(34)*, have an extensive differentiation capacity that implies not only versatility on the lineages they could derive, but also a risk of uncontrolled differentiation and potential tumor formation in envisioned clinical applications *(35–37)*. In addition, their procurement from human embryos involve relevant ethical concerns that hinder their utilization in regenerative approaches *(38)*.

As an alternative to such ethical issues, the discovery of the possibility to reprogram adult somatic cells into induced pluripotent stem cells (iPSC) *(39)*, provided a cell source that was considered more viable than ESC. However, several limitations regarding phenotype stability and safety of the mechanisms used for inducing its pluripotent status, have restricted their application in clinical scenarios *(40)*.

In contrast, ASC - more commonly acknowledged as mesenchymal stromal cells (MSC) -, do not imply major ethical issues and although with a more limited potency than ESC or IPS, represent a cell source of easy isolation and with sufficient proliferation and differentiation capacities for being used in regenerative medicine applications like bone, cartilage *(41)*, muscle *(42)* and neural tissues *(43)*. Currently, these adult stem cells can be derived from a variety of tissues, as is the case for human bone marrow *(44, 45)*, fat *(46, 47)*, umbilical cord blood *(48, 49)*, liver *(50)*, muscle *(51)*, pancreas *(52)*, blood *(53)* and brain *(54)*.

These MSC have one essential limitation laying on the heterogeneity of their populations *(55)*. Consequently, current efforts are focused on the identification and characterization of specific subpopulations that may be more suitable for the different targeted tissues *(56–58)*. Nonetheless, despite of continues efforts for consolidating a precise definition of stem cell subtypes by their properties and markers (i.e., adherence to plastic, specific surface-antigen expression and differentiation potential), this issue remains at the level of a philosophical question *(59, 60)*. Instead, their identity is now considered in the context of the specific microenvironment hosting them (i.e., the stem cell niche), categorically associated with the origin of the lineages they are capable to differentiate into, as well as their functionality both in vitro and in vivo *(61, 62).*

In this framework of disadvantageous heterogeneity in MSC populations, tissue specific progenitors stem cells (TSPSC) emerged as a novel cell type of interest for tissue engineering *(63)*. The potential of such progenitors, defined by their higher efficacy in lineage-specific differentiation, is being explored in the frame of multiple applications such as corneal, intestinal and pancreatic repair *(64–66)*.

Considering all described possibilities and with the perspective of translational applications, the primary interest of the TE field is currently focused in standardization of MSC handling, optimization of protocols for TSPSC, and identification of cell sources close in lineage to the one envisioned; for example, cells isolated from the same targeted tissue *(17)*. This alternative has the advantage of not implying ethical controversies, and the potential of give rise to stable phenotypes under appropriate conditions *(67, 68)*.

Refinement of the combination of scaffolds, cells and factors used for a particular aimed tissue, constitute the fundamental principle of current TE investigations, and its evolution is conditioned by the progressive understanding of the biology for each cell type or tissue.

In this thesis the cartilaginous tissue is the target, therefore, in order to identify the critical elements that must be here considered in view of a tissue engineered approach, its main features and characteristics are presented in the subsequent sections.

3.2 Cartilage

3.2.1 Structure and properties

Cartilage is a form of connective tissue present in different body compartments such as the nose, the rib cage and the articulations. It comprises a very specialized extracellular matrix (ECM) along with the resident cells, namely the chondrocytes. This tissue is formed during embryonic development, upon migration and aggregation of undifferentiated mesenchymal cells from the lateral plate that condensate into a cartilaginous anlage. Within this, two distinct populations of chondrocytes arise, one that will differentiate towards growth plate chondrocytes (i.e., cells that further mature into hypertrophic chondrocytes, ultimately die and are replaced by bone cells); and a second that instead, will differentiate into stable chondrocytes *(69, 70)*.

Cartilage is avascular, one of the reasons why the composition of its ECM is critical for the survival and maintenance of the chondrocytes, which in turn, produce and maintain the cartilaginous matrix. The whole tissue is solid, firm and enriched in proteoglycans and collagen fibers. This structure allows not only the diffusion of substances within the tissue, but also the withstanding of relevant mechanical effects according to the different locations where this tissue is present. The diverse mechanical properties exposed, consistent with a differential molecular composition of the ECM, enables the classification of cartilage into three main types: hyaline (compression resistant), elastic (flexible support) or fibrous (deformation resistant) *(71, 72)*. Of particular interest for my dissertation is the hyaline cartilage, located in the articular surfaces (i.e., the structures that joints

two or more bones), the nasal cavities and the fetal skeletal tissue among other body compartments (Fig. 2A).

In the knee, articular cartilage covers the surfaces of the femur, tibia and patella, conferring stability to the whole joint along with muscles, bones, ligaments and synovial tissue (Fig. 2B) *(73)*. Its main functional feature is to provide cushioning and frictionless motion to the whole structure.

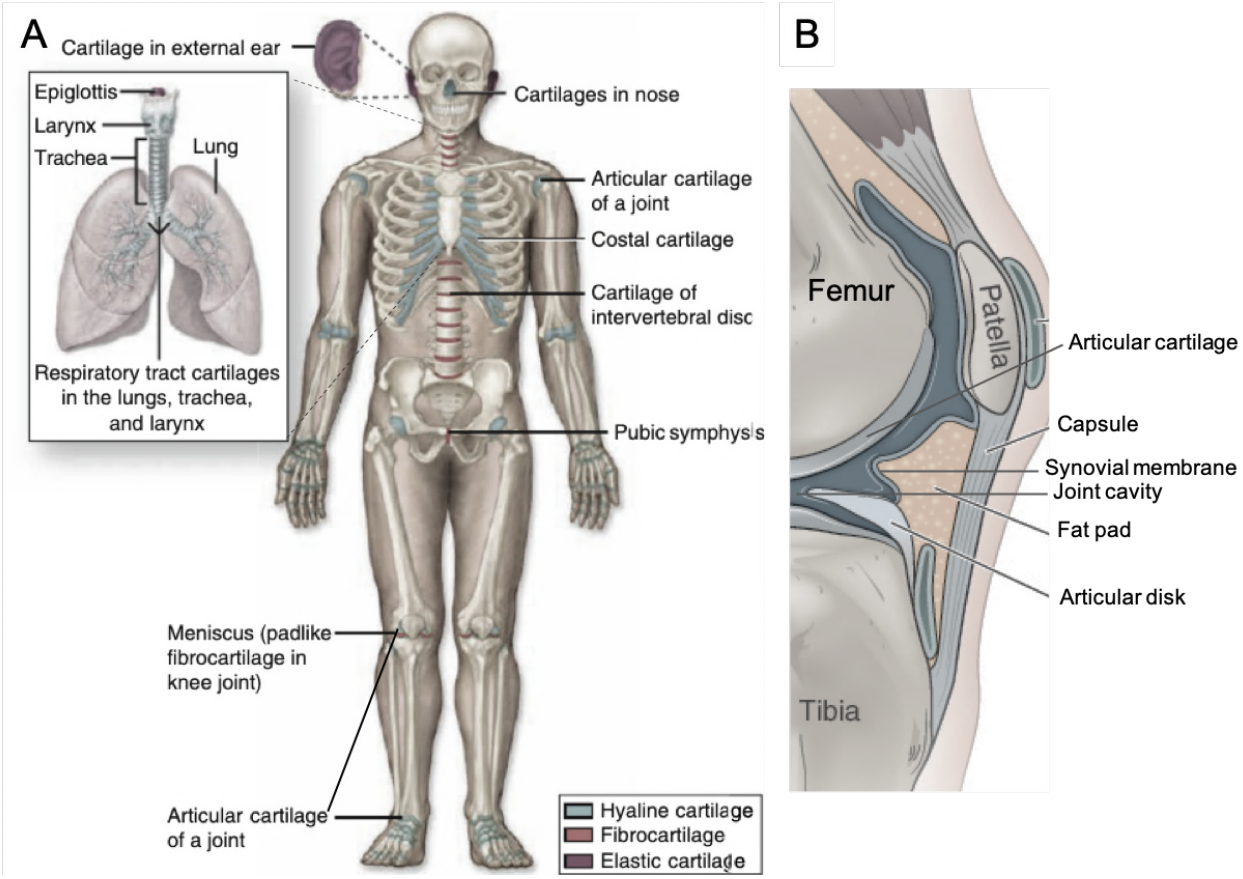

Figure 2. Cartilage subtypes in the articulations and structure of the knee. (**A**) Location of cartilage types in the human body, discriminating tissues with differential mechanical properties: hyaline (blue), fibrous (red) or elastic cartilage (purple) (adapted from *(71)*). (**B**) Anatomical configuration of the knee joint showing the articular (hyaline) cartilage at the end of the long bones (femur and tibia), surrounded by the joint cavity and covered by the synovial membrane. Other joint structures as patella, capsule and fat pad are also indicated (adapted from *(71, 74)*).

3.2.2 ECM composition

The hyaline cartilage is characterized for having low cellularity (chondrocytes are present between 2 to 5% wet weight), and a highly hydrated ECM (from 60 to 80% of water) enriched in collagens and proteoglycans, aside from other non-collagenous proteins *(40)* (Fig. 3A). The collagen fibers, as the main component of the matrix, account for 15% to 22% of its wet weight. The more predominant collagen is the type II, but other types comprising I, III, V, VI, XI and XII are also present in lower proportions. Fibrils of this extracellular component transition from thin and parallel to the surface in the more superficial zone, towards thick and perpendicular at the deeper region. This intricate gives the form and tensile strength to the tissue, in combination with other elements of the ECM that help stabilizing the network *(76)* such as the proteoglycans (PG), constituting about 10 to 15% of the tissue.

Those PG are formed by chains of sulfated glycosaminoglycans (GAG) – typically chondroitin sulfate - linked to a core protein – typically aggrecan - and agglomerated around a backbone of hyaluronic acid (HA), which is a long linear GAG. These arrangements occupy the space in between collagen fibrils (Fig. 3B), and allows the dynamic absorption or desorption of water molecules, giving the load bearing properties to the joint *(77–80)*.

Additional ECM components such as non-collagenous proteins, are also relevant for the functioning of the joint. Among these, some of the more relevant are lubricin, that as indicated by its name has a lubricating role in the articulation *(81, 82)*; fibromodulin, that interacts with the collagen fibers; and different adaptor proteins from the families of matrillins and thrombospondins (TSP), that mediated the interactions between collagen fibrils and the aggrecan network *(83)*. Among this last category, the cartilage oligomeric protein (COMP) *(84)*, member of the TSP family, denote a particular interest since it is shown to play a role in skeletal genetic disorders and diverse pathological conditions *(85)*.

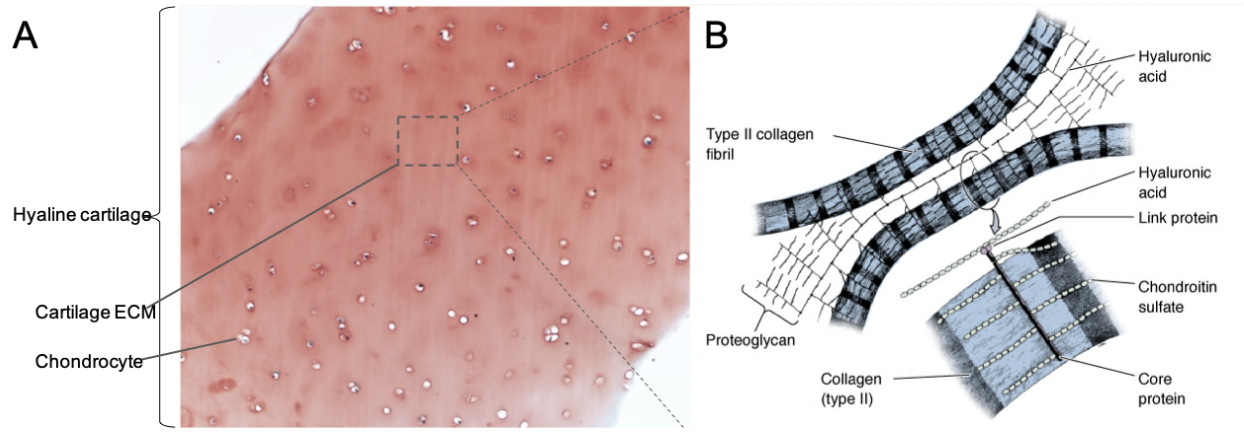

Figure 3. Structure and composition of the extracellular matrix in the hyaline cartilage. (**A**) Section of hyaline cartilage from an articular joint, illustrating the distribution chondrocytes dispersed within the highly hydrated matrix. Red color in the matrix indicates high content of glicosaminoglycans (GAG) stained by Safranin-O. (**B**) Main components of the hyaline extracellular matrix showing the arrangement of proteoglycans (PG), formed by changes of sulfated glycosaminoglycans (GAG), linked to a core protein and agglomerated around a backbone of hyaluronic acid (HA). This intricate interacts with the collagen matrix, mostly composed by fibers of type II collagen.

3.2.3 ECM maintenance

Due to the relevant composition and structure of the cartilaginous ECM, different cell-mediated processes are relevant for the preservation of this specialized network, achieved through an equilibrated remodeling of the matrix *(72)*. In short, this process involve the synthesis of degrading enzymes, as well as the deposition of new ECM molecules by the chondrocytes *(86)*.

Two main families of proteases are involved in the catabolic process, the matrix metalloproteinases (MMPs), and the 'disintegrins and metalloproteinases with thrombospondin motif' (ADAMTS). In the case of MMPs, 7 types have been shown to be expressed in articular cartilage, from which MMP1,-3 and -13 are within the more studied in the context of hyaline cartilage, because of their capacity to degrade cartilaginous ECM components including type II collagen, and their upregulation in pathological conditions *(87)*. Concerning the ADAMTS family, 19 members are

identified in human, categorized according to the substrates they are able to breakdown. In articular cartilage, ADAMTS4 and -5 are the more studied, mostly due to their aggrecanase function *(88)*.

In terms of factors promoting of the anabolic processes, TGF-β is considered the main morphogen playing a role in the synthesis of cartilaginous ECM molecules including proteoglycans and collagen, through different mediators *(86, 89)*. This factor is also necessary for the proper formation of articular cartilage at early stages of development, and its deficiency linked with multiple pathological phenotypes of the musculoskeletal system *(90, 91)*. A more comprehensive description of factors promoting cartilage formation, as well as their associated roles, is presented in the *section 3.2.7.2*.

In normal conditions, the homeostasis of the whole structure is maintained because of the balance between synthesis of matrix components and degradative enzymes. However, an alterations in any of the ECM component or in the phenotype of the joint cells cause a disparity in the metabolism, that may evolve into cartilage damage and further development of pathological conditions *(71)*.

3.2.4 Cartilage damage and therapeutic options

As stated before, the tissue homeostasis is disrupted upon events such as trauma, mechanical misalignment, genetic factors causing structural and functional damages, or any other potential lesion trigger *(92)*. On adults, the intrinsic repairing capacity of cartilage is limited, and existing lesions can remain non- or mis-healed if not treated. Clinically, this causes pain and physical disability on individuals that might ultimately generate a degenerative joint environment.

Two main types of lesions can be distinguished in the articular cartilage: *Focal* and *degenerative*. In focal injuries, the damage is restricted to a well limited area and usually a consequence of trauma *(93)*. In degenerative cartilage lesions instead, both the area and the depth of the lesion are progressively enlarged with the occurrence of additional damage, triggered by a non-corrected

mechanical or metabolic imbalance *(92)*. In both cases, when only the cartilaginous – and no the subchondral bone - layer is involved, the capacity of the tissue for spontaneous healing is very limited. In cases where the subchondral compartment is affected and stromal cells may reach the cartilage, the defect is filled up with fibrous cartilage, lacking the properties of the hyaline one *(94)*.

For the treatment of symptomatic degenerative defects, a set of strategies is used in order to relief pain or resurface the articulation. Among the available, the so-called "conservative" therapies, consisting on the administration of analgesic and nonsteroidal anti-inflammatory drugs (NSAIDs) *(95)*, are merely palliative. Main limitations of this approaches, reside on the variable responses between patients, the limited potency of the effects and the non-indicated long term usage *(96)*.

Additional pharmacological management of the pain, involves the minimally invasive intra-articular injections of glucocorticoids to locally maximize the anti-inflammatory effect, or of hyaluronic acid, to ameliorate the shock effect and lubricate the joint *(97)*. In the first case, only short term benefit of the treatment has been observed, and its usage controversial due to potential risks of adverse events concerning infections and cardiovascular events even at low dosages *(98, 99)*. Regarding hyaluronic acid injections, they are widely used in the clinics due to its effective and temporary pain reducing outcomes. However, lack of evidence about optimal formulation and dosage, limits the understanding of its real effects and mechanism of action *(100)*. This lack of knowledge, along with the limitations on assessing effects on symptoms other than pain; make necessary the discovery, development and optimization of alternative therapeutic agents such as potentially able of delaying/stopping (i.e., modifying) the progression of posttraumatic lesions towards degenerative ones. Such are commonly referred as disease modifying drugs (DMD), and are the current goal of several investigations *(101)*.

When structural restoration of the articulation is considered, surgical interventions constitute the therapeutic alternative. In the first line of treatments, removal of the excess fluids such as synovial effusion and loose bodies by lavage, or extraction of abnormal tissue protuberances by debridement, are performed with demonstrated improvement of symptoms *(102)*. For stimulating the intrusion of bone marrow into the cartilage, and therefore the formation of fibrotic tissue, strategies as bone drilling and microfracture are used *(103)*. When the reconstruction of areas with damage in both cartilage and bone, the osteochondral autograft/allograft transplantation (OCT/OAT) is the choice *(104)*.

In order to pursue the possibility of addressing further symptoms beyond structural restoration and pain reduction, alternative treatments must be used. Among those, cell-based strategies have been used as promising for non-degenerative lesions, and due to its relevance for this dissertation, an overview of the available options is presented in the next section.

3.2.5 Cell-based therapies for cartilage repair in non-degenerative lesions

In the case of focal lesions, the autologous chondrocyte implantation (ACI) is a strategy commonly used. This technique became popularly used for the treatment of full thickness cartilage defects in the 90's, and consist on the isolation of articular chondrocytes (AC) from an unaffected area, to be further expanded and injected into the defect, under a flap of periosteum - layer that covers the bony structures - as a physical barrier supporting cell retention *(105)*.

As starting point, the usage of cultured cells instead of tissue specimens from the same patient, was advantageous regarding the procurement of high amount of cells for the interventions, overcoming restrictions in source availability *(106)*. However, follow up of treated patients in the following years, evidenced some drawbacks of the technique such as detachment of the repair tissue (delamination) and periosteal abnormal growth (hypertrophy) *(107)*. In addition, other deficiencies

on differentiation instability, poor integration, cell loss and matrix degradation were identified as the principal causes for treatment failure. The logic subsequent step was the introduction of a scaffolding material instead of the periosteal flap, providing better support and mechanical stability to the graft. This strategy, recognized as the "second generation" ACI *(108)*, opened the door towards the exploration and assessment of different resources, being the collagen-based matrices the more satisfactory in terms of repair capacity and no development of hypertrophy *(107)*.

Further refining of the technique led to the direct implantation of a scaffold pre-seeded with cells, approach known as Matrix-based ACI (MACI) or "third generation" ACI *(109)*. This change of strategy offered considerable improvement regarding graft stability and handling, and is now the standard cell-based approach used for the treatment of focal lesions of limited size (< 4 mm^2) *(110)*. The more common matrices used in the diverse ACI approaches are listed in Table 1.

Table 1. Scaffolds used in cell-based approaches for cartilage repair. Comercially available matrices used for the different generations of autologous chondrocyte implantations. (Original table from *(107)*)

Technique	Product	Company
1 Generation	ACT-Biotissue	BioTissue
Cell suspension with	Carticel	Genzyme
periosteum	ChondroCelect	Tigenix
	Chondrotransplant	Codon
	Novocart	Tetec
2 Generation	Artrocell	Cellgenix/Geistlich/ Ormed
Cell suspension with collagen-membrane	CACI	Verigen
3 Generation		
Cell – biomaterial		
Hyaluronan polymer	Hyalograft	FAB
Collagen-gel	CaRes	Ars Arthro
Collagen-membrane	ArhtroMatrix	Arthrex/Orthogen
	MACI	Verigen/Genzyme
	MACT	Igor/André
Polymer	BioSeed-C	BioTissue
	Novocart3D	Tetec

3.2.6 Remaining challenges of the available treatments for cartilage repair

In all mentioned cell-based treatments, the need of causing additional morbidity in a tissue with already limited capacity for self-repair was not ideal *(111, 112)*. Moreover, none of the operative procedures (merely surgical or cell-based) can reproduce the formation of hyaline cartilage, and their outcomes are largely variable according to the severity, etiology and size of the lesion. Other patient-related factors such as gender, age and physical activity affects the outcomes too *(104)*. Because of these limitations and response-variability, no treatment can faithfully repair the structural and biological conditions of the tissue in long-term, thus allowing the progressive degeneration of the knee. In those cases - in the stages of worse severity -, the partial of total replacement of the joint by a prosthesis is required, as a merely structural solution. This last intervention, is only indicated for elder patients due to its associated higher risk for revision (i.e., the need of a second surgery) *(113, 114)*.

With this panorama, existing strategies (both pharmacological and surgical) have limitations in fully restoring healthy conditions of the joint, and there is a lack of treatment alternatives for the management of large size defects - since ACI has a size restriction - as well as for degenerative ones in young patients – since prosthesis implantation is only indicated for elder patients - *(97, 114)*.

As a conclusive remark, it is imperative to explore alternative therapies capable of filling up the gap in between treatments and patient needs. In this thesis, the development of a tissue engineering approach for the treatment of degenerative cartilaginous lesion is intended, and therefore, some considerations regarding the elucidation of TE strategies for cartilage repair, are presented in the following section.

3.2.7 Tissue engineering approaches for cartilage repair

Considering the described limitations of available surgical interventions, TE strategies have been explored in order to further optimize techniques and outcomes, as well as to develop therapeutic alternatives for the treatment of large or degenerative defects, especially in young patients *(115)*. In particular, the overcoming of two main drawbacks is perused with the implementation of tissue engineering strategies; the need of extracting relative large amounts of tissue from the joint that might not be even available in large-size or degenerative defects *(112)*, and the lack of structural support in the intervened area, potentially causing delamination and periosteal hypertrophy *(107)*. Identifying an optimal combination of scaffolds, bioactive factors and cell sources to address those challenges, is critical to achieve such aim *(116)*.

3.2.7.1 Scaffolds for cartilage tissue engineering

The more explored cell supports for cartilage TE are the ones used for autologous chondrocyte implantation (ACI), i.e., materials based of collagen, hyaluronic acid and polymers; since they have been already tested in clinical applications *(107)*. However, such materials offer a limited capacity to allow microstructural organization in terms of ECM arrangement (*section 3.2.2*), and therefore, the finding or development of more biomimetic ones is desired *(117)*.

Aiming for the replication of the biomechanical properties of the articular cartilage, hydrogels represent a suitable biomaterial to recapitulate the liquid/solid composition of the tissue *(118)*, allowing further optimization of parameters such as density, degradation and stiffness *(119)*. Instead, when properties regarding compartmental organization of the tissue are pursued, the design of scaffolds mimicking ECM native structure is the contemplated strategy to favor specific cell arrangement *(120, 121)*.

None of the approaches above mentioned has been successfully implemented in clinical scenarios, and their usage mostly restricted to in vitro investigations *(117)*. Still, for such in vivo studies, a scaffold-free cartilaginous tissues can be alternatively used as a model, by the formation three-dimensional (3D) cell aggregates under adequate chemical stimulus, involving a defined chondrogenic medium *(122–124)*. This cartilaginous micro-masses (also called pellets) can be used as individual entities *(125)* and in combination with other materials to produce larger constructs *(126)*.

3.2.7.2 Bioactive factors for cartilage tissue engineering

Bearing in mind the possibilities regarding cell supports, it is also relevant to establish the important factors regulating chondrogenic differentiation. The current broadly-used chondrogenic medium was established in the 1990s for 3D pellet cultures of MSC *(124)*, and its basic composition still valid. The most relevant factors involved, as well as their associated functions, are summarized in Table 2.

Apart of the components listed above (TGF-β, ITS, Glucose, Dexamethasone, ascorbic acid and Sodium pyruvate), many other cues play a role in chondrogenic differentiation. Regarding the physical ones, mechanical stimulation and the material properties of the scaffolds such as stiffness, are among the more relevant *(127, 128)*. In the case of additional chemical elements, the supplementation with small molecules, additional growth factors or specific inhibitors can be introduced, when specific functions or pathways are of interest *(129–131)*. Additional factors regulating the chondrogenesis, other than the commonly used in the differentiation media, are described in Table 3.

Table 2. Typical factors involved in the chondrogenic differentiation. Essential culture media supplements for the induction of chondrogenesis in cultured cells (modified from *(132)*).

Transforming growth factor beta TGF-β	• Key cartilage-inducing factor • Induce expression of the master regulator of chondrogenesis, SOX9, along with SOX5 and SOX6; further supporting the expression of ECM components such as aggrecan and type II collagen
Insulin/transferrin/selenium (ITS)	• Serum substitute supporting cell viability, metabolism, proliferation and biosynthesis • Mediator of glucose uptake • Enhancer of DNA synthesis and proteoglycan production • Chondrogenesis stimulating factor • Antioxidants (Due to the presence of *Transferrin and selenium*)
Glucose	• Major energy source and precursor of GAG *(24)*. • Associated with promotion of chondrogenic differentiation and inhibition of apoptosis
Dexamethasone	• Up-regulating effect on cartilage-related gene expression. In combination with TGF-β, it enhances the expression of COL II-XI, aggrecan, COMP and delays the expression of COL X (marker of hypertrophy).
Ascorbic acid	• Plays a role on stabilizing the triple helical structures of collagen
Sodium Pyruvate	• Energy source • Enhancer of the energy metabolism related to the Krebs cycle

3.2.7.1 Cells for cartilage tissue engineering

In line with the general approaches in the whole tissue engineering field, the MSCs constitute the more broadly explored cell source for cartilage engineering, due to their ample availability from multiple tissues, high proliferation capacity and ability to differentiate into chondrocytes *(132)*. Nonetheless, up to date none of the developed protocols or explored sources have been able to generate a stable long-term cartilaginous phenotype upon in vivo implantation *(133)*, thus hindering the clinical application of stem cell-derived chondrocytes *(134)*. One of the main challenges that remains, is the undesirable hypertrophic differentiation (i.e., cell enlargement and calcification of the matrix) that this cell type undergoes, unavoidably affecting the quality of the tissues in long-term *(116, 135)*. Additionally, the broad heterogeneity in phenotypes that have been

described among donors, tissue sources, and event between single cells upon clonal selection, represents a drawback for the reproducibility of tissue quality, that ultimately affects clinical outcomes (*section 3.1.3*) *(136)*.

Table 3. Chondrogenic regulating factors. Additional factors relevant in chondrogenesis.

Fibroblastic growth factor **FGF**	• Increases proliferation and proteoglycan production • Induces chondrogenic differentiation • The expression of specific members of this family is crucial for the proper cartilage patterning during embryonic development
Insulin growth factor **IGF**	• Increases proliferation and cartilaginous ECM production • Has and additive effect on chondrogenesis in combination with TGF-β and BMP
Bone Morphogenic proteins **BMPs**	• Enhance cartilage formation through SOX9, and production of ECM molecules such as proteoglycans and type II collagen • Some of the members of this family inhibit the development of hypertrophic features
Wingless/integrated-1 proteins/inhibitors **Wnt**	• Different members of this family play inhibiting or promoting roles in cartilage formation • Important for cell migration and condensation (adhesion)
Vascular endothelial growth factor **(VEGF) inhibitors**	• Given the avascular nature of cartilaginous tissues, the blockage of VEGF pathway (mostly by genetic manipulation of the cells and knockout experiments) have demonstrated to be chondroinductive/chondroprotective

Because of these barriers on the application of MSC, another explored cell source has been the targeted tissue itself, which is the articular tissue. Articular chondrocytes (AC) have a high potential for cartilage repair since they expose a mature phenotype and have been already employed in approaches such as the previously described ACI/MACI (*section 3.2.5*). However, AC undergo de-differentiation during in vitro culture, and can only partially recover the original phenotype in 3D *(137–139)*. Moreover, the need of isolating them from a healthier compartment that has already limitations in self-repair, constitutes a strong disadvantage since it has been associated with site co-morbidity, evidenced with reported cases of reduced joint functionality *(111)*, increased

cartilage injury scores *(140)* and appearance of degenerative changes *(141, 142)*, long term after tissue harvesting.

This scenario leaves a still unfulfilled need of identifying substitute locations from where to isolate cartilage in order to produce implantable tissue. In this attempt, chondrocytes from alternative sources have been considered as candidates for tissue engineering, as is the case for nasal septum *(143)*, rib cage *(144)*, iliac apophysis *(145)* and ear *(146)* cartilage. The chondrogenic capacity of this sources has been tested and, in some cases even already implemented in clinical applications, such as for auricular reconstruction *(147)*. The nasal cartilage in particular, has been explored by our laboratory, and others, as a promising cell source for tissue engineering applications. Due to its relevance for this thesis, a more detailed depiction of the nasal chondrocytes is presented in the following section.

3.3 Nasal Chondrocytes for cartilage repair

During embryonic development of vertebrates, a high regenerative potential and developmental plasticity has been demonstrated for cells with neural crest origin, which can be specifically programmed by environmental cues, for giving rise to numerous tissues and organs. Neural crest-derived cells with progenitor properties have been identified in various craniofacial adult tissues, like dental pulp and periosteum. Nasal chondrocytes (NC), represent a particular type of neural crest derived cells and several studies have indicated their large potential for tissue engineering and/or regenerative medicine purposes *(148)*.

These cells fulfill the mentioned requirements for cell sources in terms safe procurement with minimal donor site morbidity *(149)*, possible in vitro expansion and generation of stable engineered cartilage (both in vitro and in vivo) *(143, 150)* (*section 3.2.7.1*). When compared with articular chondrocytes, their superiority has been proven not only regarding their accessibility (in terms of

avoided morbidity and intervention simplicity), but also their higher proliferation rates and their reduced age-dependent capacity post-expansion to form cartilaginous tissues *(151, 152)*.

Envisioning the manufacturing of an articular cartilage substitute, NC display similar responses to mechanical *(153)* and inflammatory stimuli *(154)* than AC, showing its potential for being compatible with the articular environment. Previous studies of our group have also demonstrated that nasal chondrocytes lack the expression of specific HOX genes – feature associated with tissue regeneration capacities –, and that they are able to activate the expression of specific genes of this group, according to the HOX-profile of the site where they are implanted, such as the articular cartilage *(143)*. This characteristic could be associated with their neuroectodermal origin, different that the one from which the articular cartilage (as implantation site) and articular chondrocytes arise.

Provided these features and considering the need of alternative cell sources for TE or cell-based approaches, a subsequent stage in the field was the exploration of their usage on those clinical indications, particularly settled for treatment of focal defect *(105)*. With this goal, different animal studies have shown the feasibility of implantation of nasal chondrocytes-based engineered cartilage, in ectopic *(155)* and orthotopic compartments *(156)*. In the last model, their capacity to contribute with the formation of repair tissue was demonstrated.

In terms of in human applications, NC (in the form of tissue engineered constructs, N-TEC), succeeded in reconstructing nasal alar lobule after tumor resection *(149)* and, the feasibility and safety of utilizing them for the repair of focal lesions in articular cartilage defects, demonstrated in the context of a phase I trial performed by our group *(157)*. The efficacy of this treatment is being currently investigated in a phase II study (BIOCHIP, http://biochip-h2020.eu).

This approach has so far been only assessed in the context of focal (post-traumatic) lesions, but its potential extension towards the treatment of degenerative cartilaginous defects is unknown. The

feasibility of this extension in the context of a degenerative joint disease, namely osteoarthritis, constitute the pillar of this thesis. A comprehensive depiction of this pathology is presented in the subsequent segment.

3.4 Osteoarthritis (OA)

3.4.1 Definition and Symptoms

This pathological condition is the cause of about 50% of the worldwide musculoskeletal disease burden and affects 30% or the elder population *(158)*. It is triggered by an imbalance of the structure and metabolism of the joint as a whole, i.e., concerning not only the cartilage but also the adjacent tissues; the synovial membrane and the subchondral bone. The pathology is broadly defined as a progressive degeneration of the articulation, that causes pain and stiffness as main symptoms, and leads ultimately to disability *(159)*.

Commonly described morphological changes involve progressive loss of the articular cartilage, cartilage calcification, subchondral bone remodeling, and mild to moderate inflammation of the synovial membrane *(160, 161)*. The severity of this findings, which is mostly estimated radiographically or with magnetic resonance imaging, can be graded by means of different established scores considering the presence of osteophytes (bone spurs around the articulation), joint space narrowing, sclerosis (bone stiffening) and cartilage deformation *(162)*. Although various scores are generally accepted, any is recognized as a standard, and the grading of the pathology more frequently performed according to local guides of the different clinics or medical societies, hindering the possibility of consolidating a clinical picture for the disease.

For a more thorough disease diagnosis, contribution of external conditions such as mechanical injury, physical activity, aging, obesity, metabolic disorders and gender susceptibility must be

considered, as well as the possible existence of symptomatic manifestations of OA, without radiographic indications of morphological damage *(158)*.

Regarding biological events, the precise underlying mechanism for the initiation onset are not yet elucidated, but several factors converging on transcriptional regulation of inflammatory and catabolic cues, are identified as fundamental for the development and progression of disease. This, ultimately triggering cartilaginous ECM breakdown among other morphological joint alterations. Of relevance for my thesis are these inflammatory and catabolic components. For this reason, a more detailed description of their role in the pathology of OA is presented in the following sections.

3.4.2 Inflammatory component

Inflammation is a major factor associated with the risk of both biological and symptomatic manifestations of the disease, such as the cartilage loss as well as the occurrence of pain, swelling, and stiffness *(106)*. In the joints, inflammation can be observed at the macroscopic level trough evident enlargement of the synovial membrane, due the infiltration of inflammatory cells *(163)*; but also, with the presence of synovial effusions caused by an increased secretory activity of the cells in the joint tissues *(159)*. More recently, a chronic 'low-level' inflammation, referred as micro-inflammation, has been highlighted as relevant for the disruption of the joint homeostasis and the triggering of degenerative responses *(164)*.

Pro-inflammatory cytokines such IL1β, TNFα, IL6 and IL8, have been extensively investigated, both for their in vivo role and their potential for inducing OA features on in vitro setups *(165)*. These inflammatory factors have been showed to induce an increased expression of matrix degrading proteins, including matrix metalloproteinases (MMPs) and various types of a disintegrin and metalloproteinase with a thrombospondin type 1 motif (ADAMTS) *(166–168)*, as well as to down-regulate the expression of anabolic markers such as type II collagen and aggrecan *(106)*.

No defined way of action has been yet depicted for the different factors, and its elucidation is rather complex considering that multiple cell types are able to produce them and respond to their signaling *(169)*. Nonetheless, abundant evidence about the effects of some of the more studied factors in the joint metabolism has been reported. In this regard, relevant facts for this thesis summarized below.

3.4.2.1 IL1β

This cytokine is considered one of the key factors involved in the pathogenesis of OA. It induces inflammatory reactions and catabolic effects independently, as well as in combination with other mediators *(165)*. It is produced by the chondrocytes, but also by osteoblast (OB), synovial cells and other cells that infiltrate the synovial membrane during an inflammatory event *(170–172)*. In OA patients, IL1β levels are increased in synovial fluid, synovial membrane and cartilage, as compared to healthy samples *(173, 174)*. In the case of OB isolated from osteoarthritic subchondral bone, IL1β levels do not differ from those detected in healthy donors *(172, 175)*.

Different studies have described the effects of IL1β in a reduced synthesis of ECM components by the chondrocytes, such as type II collagen and aggrecan *(176, 177)*. In addition, the cytokine also enhance the production of ECM degrading enzymes including MMPs and ADAMTS4 *(165, 178–180)*. As additional effects, IL1β regulate its own secretion by autocrine signaling, additionally stimulating the production of other cytokines such as IL6, IL8, CCL5/RANTES and TNF*α (181–184)*.

The role of IL1β is not only associated to the OA onset, but also to other pathological conditions like diabetes *(185)*, and relevant cell processes like senescence *(186)*. It also has a synergic effect in combination with TNF*α (187),* cytokine that shares some of the IL1β functions in osteoarthritis, as explained in the following section.

3.4.2.2 TNFα

Tumor necrosis factor alpha (TNF*α*), along with IL1β, is considered a relevant inflammatory cytokine involved in the pathological processes in OA. It is secreted by the same cells in the joint than IL1β, and similarly to the effects attributed to this last one, its increased concentration is also observed in joint tissues and synovial fluid in samples of donors affected by the disease *(170–172, 174)*. Moreover, higher levels soluble receptors of TNF*α* (TNFsR1 and TNFsR2) have been correlated with increased pain, stiffness and physical disability in patients with knee OA *(188)*.

The effect of TNF*α* coincides, in most cases, with the action of IL1β such as in the impairment of the metabolic activity towards a reduced production of ECM components including proteoglycans and type II collagen *(189, 190)*, and an increased secretion of pro-inflammatory (IL6, IL8, RANTES - regulated on activation, normal T cell expressed and secreted -) and degrading molecules (MMPs and ADAMTS) *(139, 191)*. IL1β and TNFα together, induce the secretion of other pro-inflammatory cytokines such as IL17 in the cells that infiltrate the synovial layer, and IL18 the cells from the joint tissues *(106, 165)*.

3.4.2.3 IL6

This cytokine, usually produced in response to IL1β and TNF*α,* is secreted by chondrocytes, synovial cells, cell infiltrates from the synovial layer, osteoblast and osteochondral tissues isolated from osteoarthritic joints *(174, 175, 192, 193)*. In chondrocytes, osteoblast, synovial fluid and synovial tissues from OA donors, IL6 levels are higher, in comparison to healthy samples. In addition, concentrations of IL6 are considerably elevated in the synovial fluid from symptomatic cartilage defects, and comparable to levels measured in OA samples *(192, 194–196)*. Moreover, detection levels correlate with the severity of the lesion, when assessed radiographically *(197)*.

The effects of IL6 on the joint converge with those exerted by IL1β and TNF*α*, decreasing the production of type II collagen and increasing synthesis of degrading enzymes, specifically MMPs

in chondrocytes and osteoblast *(198–200)*. Nonetheless, its concentrations are of higher order of magnitude than the commonly reported for these other two cytokines, facilitating its detection by different analytical setups *(171, 174)*.

3.4.3 Catabolic imbalance

As part of the metabolic activity of the articular cartilage, catabolism of the ECM is performed by degrading the enzymes with collagenase and aggrecanase activity, MMP1, -3 and -13 as well as ADAMTS4 and -5 (*section 3.2.3*). In pathological scenarios such as OA, circulating levels of this enzymes are increased, turning the environment predominantly catabolic *(161)*.

The mentioned MMPs are detected in the synovial fluid of patients with OA, along with pro-inflammatory factors like IL6 *(201)*. Studies have mostly focused on the role that MMP13 have in this pathology, demonstrating the correlation of increased levels of the enzyme with the appearance of early OA signs *(202)*, as well as the feasibility of partially rescuing the phenotype with its depletion *(203)*.

ADAMTS4 and -5 as the more studied members of their family, have been also associated to the onset of OA, for being detected at higher levels in samples from patients with OA signs than in healthy controls *(204–207)*. Being less characterized than MMPs, contributions of the individual enzymes to the disease progression are not yet understood. However, it is known that deletions of this two members - ADAMTS4 and -5 - can prevent ECM degradation and OA progression in a mouse models *(208, 209)*. Other members of the family might be also involved in the development of the OA phenotype, and their role is currently studied in several investigations *(210)*.

3.4.4 Alterations in cartilage-surrounding tissues

Occurrence of OA, in different degrees of severity, implies inflammatory responses and morphological changes in all joint tissues; including articular cartilage, synovial membrane and

subchondral bone. Regarding the manifestations of those processes, there is a spectrum of cellular and molecular phenotypes that varies both between stages of the disease and affected tissues *(211)*.

3.4.4.1 Synovial membrane

Several studies have reported histological evidence of an inflammatory cell infiltrate on synovial tissues isolated from OA patients. This inflammation, called synovitis, refers to the thickening of this tissue, mostly due the presence of cells such as macrophages and T cells *(160)*. Reported inflammation levels in OA samples - considering histological findings and transcriptomic analysis -, are higher than in healthy controls but lower than in other inflammatory cartilage diseases such as rheumatoid arthritis. This, emphasizing the more micro-inflammatory conditions in OA *(171)*. The presence of inflammatory cells in this tissue, increase its secretory activity and results in synovial effusions that may be visualized by magnetic resonance imaging (MRI) or ultrasound, and that correlate with the presence of pain and dysfunction in the articulation *(106)*. Such effusions contain several pro-inflammatory, pro-angiogenic and 'pro-repair' cytokines that are elevated in joints with symptomatic cartilage defects and/or osteoarthritis, in concentrations that vary according to the pathological status *(174, 212)*. The circulating molecules then, signal trough the cells they reach – chondrocytes, osteoblasts or synovial cells itself – and trigger the catabolic cascade described in *sections 3.4.3* and *3.4.4*.

3.4.4.2 Subchondral bone

Articular cartilage is normally avascular, aneural and separated from the subchondral bone by a zone of calcified cartilage. Appearance of fissures in this barrier enable vascular invasion into the calcified cartilage, and cross-talk between the two tissues through diffusion of small molecules *(213)*. Regions of vascular invasion are associated with localized bone marrow replacement by fibrous tissue - which is use as therapeutic approach *(103)* -, but also with the amplification of the

pathological signals, while allowing the infiltration of inflammatory cells into the marrow spaces and the cartilage *(214)*.

Some morphological changes include an increased bone density and protrusions of bone into the cartilage layer, leading to cartilage tightening and contributing to the stiffening and biomechanical impairment of the articulation *(215, 216)*. As the pathology progresses, the coupling of bony outgrowths with cartilaginous damage, allow the secretion of fluid, cells and molecules towards the joint cavity and between all joint tissues *(217)*. The subchondral bone itself contributes to the production of inflammatory and catabolic factors *(175, 193)*, but it also responds to this environment by modulating its genomic and secretory profiles in reaction to the circulating molecules *(175, 218, 219)*.

3.4.5 Therapeutic options for OA defects

Considering the variety of etiologies, symptoms and anatomical distributions, no standard medical management is stablished and used approaches individualized instead. Nonetheless, up to date there is no available treatment capable of stopping the progression of the disease or recovering the properties of the lost/damaged hyaline cartage *(159)*, and the existing pharmacological and surgical approaches are mainly palliative.

A general line of intervention levels is defined, according to the severity of the disease. As first measure, behavioral interventions such as physical activity or dietary changes are recommended to patients in order to strengthen the leg structures and reduce body weight; thus decreasing the mechanical load and preventing further damage *(158)*. The following approach, which can be combined with the first one, consist on the provision of analgesic and/or anti-inflammatory drugs (non-steroidal), to later pass to the intra-articular injections of hyaluronic acid or corticosteroids, as the general conservative treatment for all cartilaginous lesions (*section 3.2.4*). In cases of worse

severity, the non-reversible (partial or total) replacement of the knee by a prosthesis is the last therapeutic option *(113, 114)*.

In general, a combination of strategies must be considered in order to achieve better outcomes, such as the tailoring of the approaches in accordance with patient conditions, co-morbidities, risk factors or individual perception of health status *(159)*. Additionally, it is important to take into account that the scope of the treatments is significantly conditioned by the physical activity as well as by the post-intervention rehabilitation plans *(220)*.

In this pathological condition (OA) the treatment with cell-based approaches, such as the stablished for focal lesions, is not an option. First, evidence of degenerative signs of the joint is considered a contraindication for such procedures *(221)*. Besides, in the scarce cases they have been applied in subjects with indications of osteoarthritis, the long-term outcome for the majority of patients has been the treatment failure *(223)*, despite of some short and mid-term observed benefits *(222)*. In this context, the therapeutic challenge is to develop a therapy able to address both symptomatic and structural changes. Therefore, current efforts are focused in the development of implantable grafts capable of stopping or inhibiting the structural deterioration caused for the pathology *(159)*, and in the identification of appropriate research models that can be used to assess the clinical relevance of the new developments. An overview of the models that could be exploited for this purpose are described below.

3.4.6 Models for the study of osteoarthritis

No in vitro or in vivo gold standard model is stablished for the assessment of therapeutic agents in OA. Therefore, setting up the pertinent conditions able to recapitulate some features of the disease, is one of the current goals from both scientific and clinical perspectives *(224)*. This section provides

a depiction of the in vitro and in vivo approaches commonly followed in the field, towards the development of representative models.

3.4.6.1 In vitro

Laboratory studies using cell-based assays as well as human OA joint tissues have identified a number of molecular pathways that are induced by mechanical, inflammatory, and oxidative stresses. In the cartilage, these stimuli results in phenotypic alterations of the chondrocytes, loss of homeostasis, and activation of abnormal cell signaling or transcriptomic profiles *(225)*.

Different models simulating those independent stresses, contemplate the employment of cell monolayers, 3D cultures and tissue explants, as well as their exposure to one or more of the stimuli above mentioned. However, considering that not precise mechanisms controlling the OA phenotype have been yet elucidated, no physiologically relevant conditions have been achieved either. Being the 3D culture system the in vitro model used in this thesis, is worth mention that apart of being capable to resemble the ECM composition of native cartilage *(226)*, it is also capable to react different inflammatory and mechanical stimuli in the context of a simulated OA environment *(125, 227)*.

Regarding inflammatory factors, the cytokines IL1β and TNFα are classical choices, knowing their widely documented catabolic effects on cartilage, and their multiple associations with OA (*section 3.4.3*). As alternative sources of pro-inflammatory molecules, the usage medium conditioned by inflammatory cells have been also used for the culture the engineered tissues *(228)*. Responses of such approaches are often assessed in regards to the histological quality of the tissues, changes in matrix composition, as well as in reduced viability and transcriptomic alterations of the chondrocytes *(229)*.

Tri-dimensional micro-pellets of primary articular chondrocytes, studied in the context of a more acute inflammation 'high dose, short exposure time', have responded to IL1β – alone or in combination with TNFα - with a reduction of GAG and an up-regulation of inflammatory and catabolic factors such as IL8, monocyte chemoattractant protein-1 (MCP1) and MMP1 and -13 *(125, 154, 230)*. Lower concentrations and prolonged exposure time have been also investigated in 3D cultures of a chondrocyte cell line, by exposing them to the typically used cytokines (IL1β and TNFα) and to macrophage conditioned medium. Such exposure leading to the upregulation of catabolic markers and reduced expression of ECM components *(228)*.

These in vitro models have been useful as a preliminary screening to elucidate the responses of chondrocytes to different factors, and are desirable for the intention of reducing animal experimentation *(231)*. However, two main limitations need to be surpassed in order to obtain clinically relevant results; the oversimplified simulation of the pathological features, and the non-physiological levels of factors and forces, often use to favor an effect. A more representative model would include not only the cross talk of biochemical cues among the different joint tissues, but also the mixed effect of the inflammatory and mechanical elements *(225)*.

3.4.6.2 In vivo

Considering the described disadvantages of in vitro models in terms of their limited capacity to recreate a physiological scenario for translational approaches, in vivo animal models provide a better platform to study OA as a disease of the whole joint, and with the possibility of incorporating the biomechanical component *(232)*. Nonetheless, it is important to take into account that no animal model is suitable for evaluating the efficacy of a potential human therapeutic approach *(224, 233)*, and only specific questions can be reliably solved by choosing the more appropriate one.

Small animal models such as mice, rats, rabbits and guinea pigs as well as large size including goat, sheep and horse, offer several advantage and drawbacks that must be considered in regards to the

application or intended outcome. Small species are often cheaper, easier to handle and analyze, and can usually give faster results. Large animals instead, lack the practical handling but are biomechanically and anatomically more similar to the human joins *(234, 235)*. Because of such differences, small animal models are more suitable for the study of pathogenesis or pathophysiology of the disease, whereas large models shall be used for pre-clinical evaluations of drugs and disease modifying agents *(236)*.

Another relevant aspect of the in vivo models is the possibility of studying naturally occurring or induced OA. In the first case, models (small and large) exposed the phenotype with aging, due to genetic pre-disposition (or modification), as well as to intrinsic species-specific properties. Its relevance lies on the fact, that it makes possible to study the stages of the disease in a non-artificial timing, according to the lifespan of the particular model *(237, 238)*. On induced OA, degenerative changes in the articulation are triggered by a direct insult to the joint, which can be surgical or chemical. The advantage of this model is that it allows the achievement of a faster OA onset, as compared to the naturally occurred ones, and that can be used to simulate a post-traumatic lesion, one of the common types of OA *(239, 240)*.

In this thesis different in vitro and in vivo models have been used, in order to evaluate the performance of nasal chondrocytes-based tissues in different OA simulating environments. The description of such models as well as the derived findings are presented and discussed in the following sections.

4 Evaluation of the compatibility of tissue engineered cartilage generated with nasal chondrocytes (N-TEC) with an osteoarthritic (OA) simulated environment.

4.1 Aim I: Responses of nasal chondrocytes-based engineered cartilage to OA-simulating inflammatory conditions

4.1.1 Introduction

Inflammation is considered one of the main triggers for the development and progression of osteoarthritis *(106)* along with the mechanical insult *(161)*. Continuous low-level secretion of the inflammatory molecules IL1β, 6, 8 and TNFα, referred as chronic inflammation *(164)*, is co-related with the production of matrix-degrading enzymes, such as ADAMTS and MMPs that ultimately cause cartilage degradation as the most prominent indicator of OA *(241)*. Those factors may be produced by the chondrocytes themselves (6, 7) or by the surrounding tissues such as synovial membrane (8, 9) and subchondral bone (10, 11). In an OA joint, the presence of these molecules would represent a challenge for any cell-based therapy upon implantation.

Consequently, being the main aim of this thesis to explore the potential application of nasal chondrocyte-based engineered cartilaginous tissues (N-TEC) for the treatment of OA lesions, I first investigated whether they can withstand a prolonged exposure to OA-simulating factors in vitro. These conditions were reproduced either by a combination of the commonly used pro-inflammatory cytokines (IL1β, 6, and TNFα), or by the secreted factors from osteoarthritic cells and tissues. The effect of such stimuli was evaluated in terms of the maintenance of the cartilaginous matrix as well as proteomic or transcriptomic alterations, in order to evaluate the performance of N-TEC in an OA-joint environment.

No cell-based strategy has been recognized as conventional for the treatment of OA-lesions yet, therefore, no standard cell type was an evident choice to be use as control and for comparison.

Nonetheless, healthy articular chondrocytes (AC) were used throughout the entire experimental setup to (i) have a reference of the cartilaginous capacity of cells from the targeted tissue (i.e., articular cartilage) and to (ii) assess whether effects were specific to the nasal origin of the cells, or a general chondrocyte-related feature.

Responses of human nasal chondrocytes to inflammation have been previously studied using this model, either in the context or an acute-like inflammation, by short exposure (3 days) to the inflammatory factor IL1β *(154)*, or in a chronified environment, by prolonged exposure to the cocktail here used (IL1β, -6, and TNFα) in combination with nutrient deprivation *(242)*. This chapter describes the performance of N-TEC under chemically defined, or physiological conditions, simulating the inflammatory component of OA in vitro, when cultured in similar settings than the stablished for clinical manufacturing of N-TEC.

4.1.2 Results

4.1.2.1 N-TEC respond to OA-inflammatory conditions with a reduction in GAG deposition and upregulated expression of inflammatory/catabolic mediators

In order to evaluate the effect of selected inflammatory conditions on N-TEC, nasal (and articular) chondrocytes were isolated and chondrogenically differentiated - in the form of 3D macropellets-. Engineered tissues were then exposed for up to 14 days to (i) low and (ii) high concentrations of a cocktail of the pro-inflammatory cytokines IL1β, IL6, and TNFα, as well as to media conditioned by (iii) osteoarthritic osteochondral tissues (OA-OC_CM) or (iv) osteoarthritic synovial cells (OA-Syn_CM) (Fig. 4A). TECs cultured without chondrogenic/inflammatory factors (in control medium, CTR) were used as control samples, and comparison with A-TEC were performed.

After exposure to the different conditions, histological characterization of the treated tissues by Safranin-O staining, evidenced a high inter-donor variability in all control (CTR) and OA-inflammatory conditions, as expected for primary cells (Fig. 4B). Indirect assessment of the glycosaminoglycan (GAG) content, performed by quantification of the Safranin-O intensity (scoring from 0=no stain to 3=dark and even stain), showed a trend for higher GAG in nasal chondrocyte-based tissues, in all conditions and time points and as compared to articular chondrocytes-based tissues. This differential quality between N-TEC and A-TEC was significant for non-OA treated samples (CTR) at both time points, and after 14 days of exposure to *High Inflammation* or *OA-Syn_CM*. Only non-inflammation treated A-TECs (CTR) were affected by the culture time, with a significantly lower GAG content at day 14, that the one measured after 7 days of culture. The more detrimental OA-inflammatory condition was found to be the *High Inflammation*, affecting significantly the quality of A-TEC tissues after the first week of treatment, and of N-TEC by the end of the culture, as compared to CTR (Fig. 4C).

For quantifying the effect of tested inflammatory conditions in the GAG content of N-TEC, the amount of sulfated glycosaminoglycans in the extracellular matrix of the treated pellets was measured and normalized by the amount of DNA; parameter that remained stable within the different experimental conditions (Table 4). Similar to previous findings, GAG content of N-TEC showed a trend towards reduction in engineered tissues treated with OA-inflammatory conditions. No significant changes were detected between baseline (GAG content prior treatment) and N-TEC, at any of the OA-simulating conditions and exposure times. Nonetheless, A-TEC underwent a significant reduction in the majority of conditions. Exposure to OA-OC_CM did not cause any significant effect on the engineered tissues, both nasal and articular chondrocyte-based. A reduction in the CTR condition, that was significant for A-TEC, did not permitted a clean discrimination of the net effect for the different inflammatory conditions on this parameter (Fig. 5A).

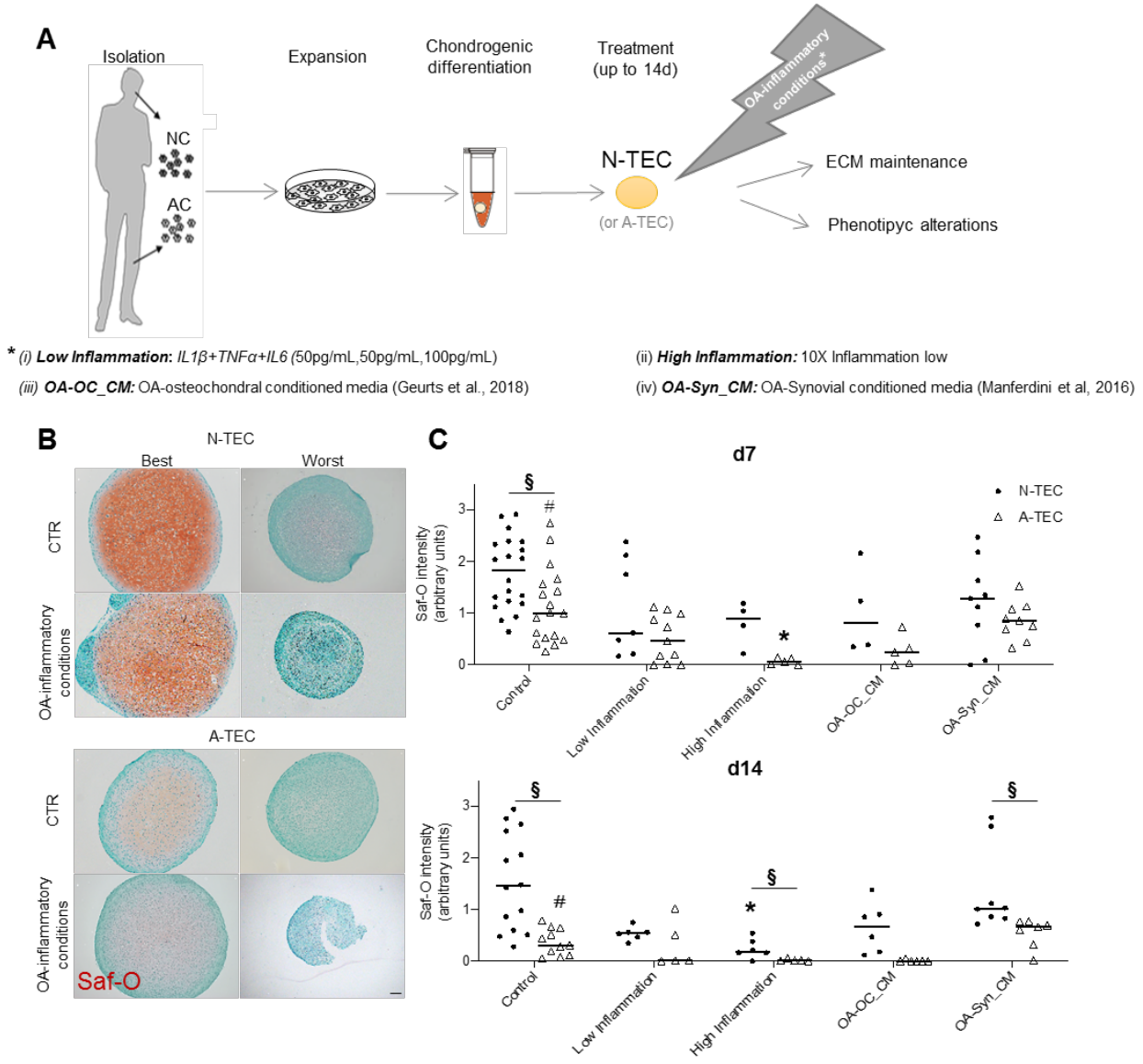

Figure 4. Responses of N-TEC and A-TEC to OA-simulating inflammatory conditions. (A) Schematic presentation of N-TEC formation and further exposure to different conditions simulating the inflammatory component of OA. (B) Histological quality of engineered tissues with nasal (N-TEC) or articular (A-TEC) chondrocytes assessed by safranin-O staining. Best and worst tissues shown for control (CTR) and all OA-inflammatory conditions by the end of the culture (d14). Scale bar: 100 µm. (C) Quantification of safranin-O staining for N-TEC and A-TEC after 7 and 14 of exposure to the different experimental conditions. The score indicates 0= No stain, 1= Weak stain, 2= Moderately even stain and 3=Even dark stain, parameters based on the "Bern Score" *(243)*. § Indicates significant differences between N-TEC and A-TEC at the same time point and condition, * from control at the same cell type and time point, and # between time points for the same cell type and condition. P <0.05 considered significant after comparison by Kruskal-Wallis and Mann-Whitney with Bonferroni correction.

Table 4. GAG and DNA content of N-TEC and A-TEC exposed to OA-simulating conditions. Quantified levels of glycosaminoglicans (GAG), DNA and GAG/DNA in the extracellular matrix of engineered tissues (N-TEC and A-TEC), after exposure to different OA-inflammatory conditions. Values represented as the mean ±SD of 6 to 20 experimental replicates per condition. Baseline values correspond to the amounts of the indicated parameter prior exposure to any condition. *Indicates significant differences with baseline for the corresponding cell type and §between cell types for the corresponding timing and condition (for an n of 6 to 20 experimental replicates per condition). Comparisons assessed by Kruskal-Wallis. P <0.05 considered significant.

		DNA (µg)						GAG (µg)						GAG/DNA					
		N-TEC			A-TEC			N-TEC			A-TEC			N-TEC			A-TEC		
d0	Baseline	1.8	±	0.9	1.2	±	0.7	11.5	±	11.6	10.4	±	11.6	6.6	±	7.7	7.9	±	6.4
	Ctr	1.6	±	1.0	1.0	±	0.7	7.5	±	7.8	4.6	±	6.4	4.7	±	4.5	3.7	±	3.0*
	Low Inflammation	1.2	±	0.9	1.0	±	0.7	5.7	±	6.4	5.1	±	6.5	7.3	±	11.7	3.3	±	2.9*
d7	High Inflammation	2.0	±	0.9	1.8	±	0.3	4.3	±	2.9	4.9	±	1.7	2.4	±	1.2	2.6	±	0.6*
	OA-OC_CM	1.8	±	0.6	1.8	±	0.2	10.6	±	9.2	7.6	±	1.1	6.0	±	5.4	4.2	±	0.7
	OA-Syn_CM	1.6	±	1.0	0.8	±	0.2	5.1	±	3.6	2.2	±	0.4	3.0	±	1.1	2.8	±	0.9*
	Ctr	1.4	±	0.9	0.6	±	0.5	5.3	±	4.9	2.1	±	2.8	3.2	±	1.7	2.5	±	1.9*
	Low Inflammation	1.8	±	0.6	1.1	±	0.4	7.9	±	5.5	4.5	±	5.2	4.0	±	2.6	4.0	±	4.3
d14	High Inflammation	1.9	±	0.7	1.0	±	0.3	4.2	±	2.1	2.3	±	0.5	2.1	±	1.0	2.4	±	0.8*
	OA-OC_CM	1.6	±	0.8	0.9	±	0.4	6.7	±	3.8	2.9	±	2.6	4.5	±	3.1	2.8	±	2.0
	OA-Syn_CM	1.5	±	1.2	0.5	±	0.2*§	4.1	±	3.4	0.9	±	0.9*§	3.4	±	1.7	2.2	±	2.7*

As a complementary analysis, the responses of the culture system in terms of gene expression profile were also evaluated. Levels OA associated factors such as *IL6, IL8/CXCL8, MMP3, MMP13, ADAMTS5* as well as the ECM-cartilage related *COL2, 1 and ACAN* (encoding genes for type II collagen and aggrecan), were screened by RT-PCR for all conditions and time points, including an earlier time point (3d). For the majority of the factors, no major trends were identified along the different conditions tested. Nonetheless, obtained data indicated a trend for increased expression of these genes in the different inflammatory conditions, as compared to baseline (expression prior treatment) or to CTR (at the corresponding exposure time).

Expressed levels of *IL6* and *MMP3* however, showed a tendency of higher up-regulation in A-TEC for the majority of conditions, as compared to N-TEC, just significantly different for *MMP3* in the *Low Inflammation* condition at d7. Particularly, in line with the findings derived from the

histological analysis, the condition of *High Inflammation* was the more detrimental one, triggering a significantly higher expression of both genes, as compared to CTR, during the first two exposure times (d3 and d7). Exposure to OA-OC_CM did not cause any significant effect on N-TEC or A-TEC, similarly to the observations obtained from the GAG analysis. No differences between times of exposure were detected (Fig. 5B).

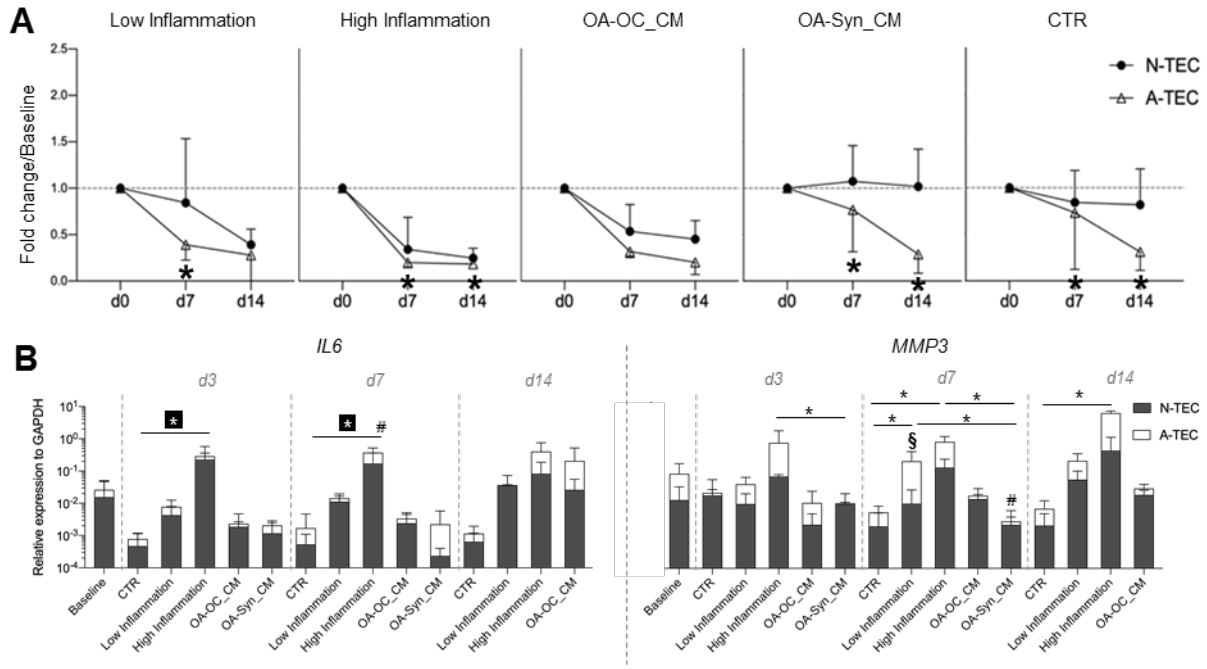

Figure 5. Effects of OA-inflammatory conditions in GAG content and transcriptomic profile of N-TEC and A-TEC. GAG/DNA measurements of treated tissues based on nasal (N-TEC) or articular (A-TEC) chondrocytes, in different OA-simulating conditions and exposure times. Dashed line indicates GAG content of the Baseline (prior treatment). *Indicates significant differences with Baseline for the corresponding cell type. (B) RNA levels of IL6 and MMP3 of treated N-TEC (and A-TEC) in different OA-conditions and exposure times. Significant differences between conditions at the corresponding timing are indicated with ■ for N-TEC and * for A-TEC, # Indicates differences with baseline and § between cell types at the corresponding timing and condition. Multiple comparisons assessed by Kruskal-Wallis, followed by with Mann-Whitney with Bonferroni correction. P <0.05 considered significant.

The findings here described for the histological quality, GAG composition and gene expression profiles of treated specimens, show that N-TEC have a superior quality than A-TEC at CTR conditions (i.e., not-inflammatory and not-chondrogenic), and indicates that this quality may be

better preserved by nasal chondrocyte-based tissues after exposure to OA-simulating conditions. Results also show that despite of the high variability between samples, engineered tissues are capable of responding to inflammatory conditions, and that this response can be followed by changes in the ECM composition such as a reduction in GAG, and by changes in the cellular phenotype such as alterations in the gene expression profile. It was additionally observed that no significant changes occurred between time points, with exception of A-TEC in CTR condition; and that *High Inflammation* was the more harmful condition while the exposure to *OA-OC_CM* did not trigger any detectable response on N-TEC (or A-TEC).

4.1.2.2 N-TEC better preserve the overall cartilaginous quality in conditions physiologically simulating the OA onset

Considering that used concentrations of inflammatory factors for the condition of *High Inflammation* are not representative of a physiological scenario, and additionally, that *OA-OC_CM* did not caused any response in the treated cartilaginous tissues; the conditions *Low Inflammation* and *OA-Syn_CM* were selected for a broader estimation of the responses of N-TEC to OA-simulating conditions.

As a complementary analysis for the quantification of the GAG content in the Safranin-O stained tissues (Shown in Fig. 4B), a system considering not only the intensity of the staining, but also cellular distribution and morphology, namely the Bern Score *(243)*, was used for a more complete evaluation of the engineered-tissue quality. This assessment showed that histological quality of N-TEC was only affected after 7 days of exposure to the condition of *Low Inflammation* (**P* =0.0171). Instead, the quality of A-TEC was significantly affected by the OA-Syn_CM at both exposure times (**P* = 0.0079 at d7 and **P*= 0.010 at d14), and this reduction was even significantly lower by the end of the culture time ($^{\#}P$ = 0.036 between d7 and d14).

Comparison between cell sources, evidenced that the quality of N-TEC was significantly higher than A-TEC in the majority of conditions, with exception of the Low Inflammation at d14 (Fig. 6A). Representative images for the quality of the corresponding tissues, stained with Safranin-O are shown in Fig. 6B. This better preservation of the cartilaginous extracellular matrix was confirmed by immuno-histological detection of type II collagen, in which this component was present in N-TEC tissues (both CTR and N-TEC treated), but absent in the A-TEC exposed to OA-Syn_CM (Fig. 6C).

To evaluate more thoroughly possible alterations in the cellular phenotype induced by the OA-simulating conditions selected, gene expression levels of different inflammatory (*IL6, IL8*), catabolic (*MMP3, MMP13 and ADAMTS5*) and cartilaginous (*COL2, ACAN and COL1*) factors were quantified at day 7. None of the tested conditions significantly regulated the expression of the tested factors in N-TEC tissues, as compared to the CTR and as assessed by qRT-PCR analysis. However, the pro-inflammatory *IL6* and *IL8*, as well as the catabolic *MMP3* were up-regulated in A-TEC upon exposure to *Low Inflammation.* No changes in the expression of cartilage related factors were detected by any condition in both cell sources. For some of the modulated factors, expression levels in tissues treated with *Low Inflammation* were significantly higher than in the OA-Syn_CM condition (*IL6* and *MMP13* in N-TEC, *IL8* and *MMP3* in A-TEC). This last condition (OA-Syn_CM), did not modulate the gene expression profile in any of the cell sources used (Fig. 7A).

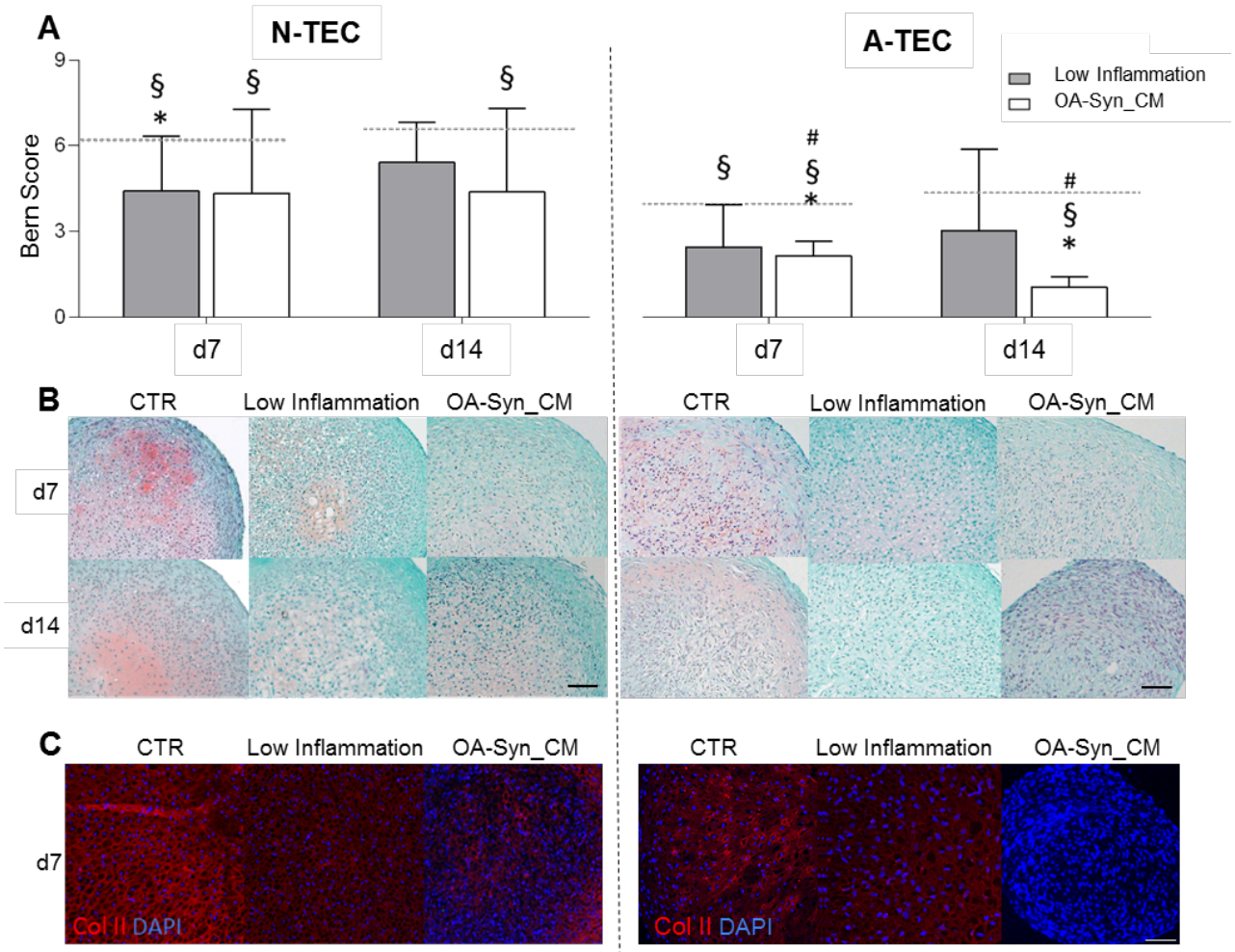

Figure 6. Cartilaginous properties of N-TEC and A-TEC exposed to *Low Inflammation* and conditioned medium of osteoarthritic synoviocytes (OA-Syn_CM). (**A**) Histological quality of N-TEC (left) and A-TEC (right) following 7 and 14 days of exposure to the selected conditions, assessed by the Bern Score. Scale represents arbitrary units from 0= lower quality to 9 = higher quality. Dashed lines represent the score of tissues in control condition (CTR). Values presented as mean ±SD for an n = 7 donors (per cell type), except for *OA_CM* (n = 5 donors). * Indicates significant differences versus CTR; # between time points and § between N-TEC and A-TEC at same time and condition, as defined by unpaired (parametric) students T-test. *P* <0.05 considered significant. (B) Staining of the extracellular matrix for GAG by Safranin O and (**C**) type II collagen (Col II) by immunofluorescent detection (at d14).

To evaluate the effects of the chosen OA-conditions in the secretory profile of N-TEC, inflammatory and metabolic factors were quantified in the culture supernatants of N-TEC and A-TEC after exposure to Low Inflammation or OA-Syn_CM (Table 5). Factors such as MIP1α/CCL3, IL12, IL13, IL17, TNFα and INFγ were not detectable in CTR but their secretion induced after the treatments. A second group of factors (i.e., MIP1β/CCL4, MCP1/CCL2, IL4, IL6, IL8/CXCL8,

IL1RA and RANTES) was already detectable in CTR, but the secreted amounts increased upon the different OA-simulating conditions. Among the evaluated panel, factors such as Eotaxin/CCL11 and IL1β were not modulated in either NC or AC by the exposure to inflammatory conditions. RANTES was the only factor for which significant differences were detected between cell sources, being up-regulated by A-TEC (and not by N-TEC) in the condition of *Low Inflammation*. The observed modulatory effects in factors classically studied by their relevance in OA (IL1β, IL6, IL8, TNFα, RANTES) are illustrated in Fig. 7B.

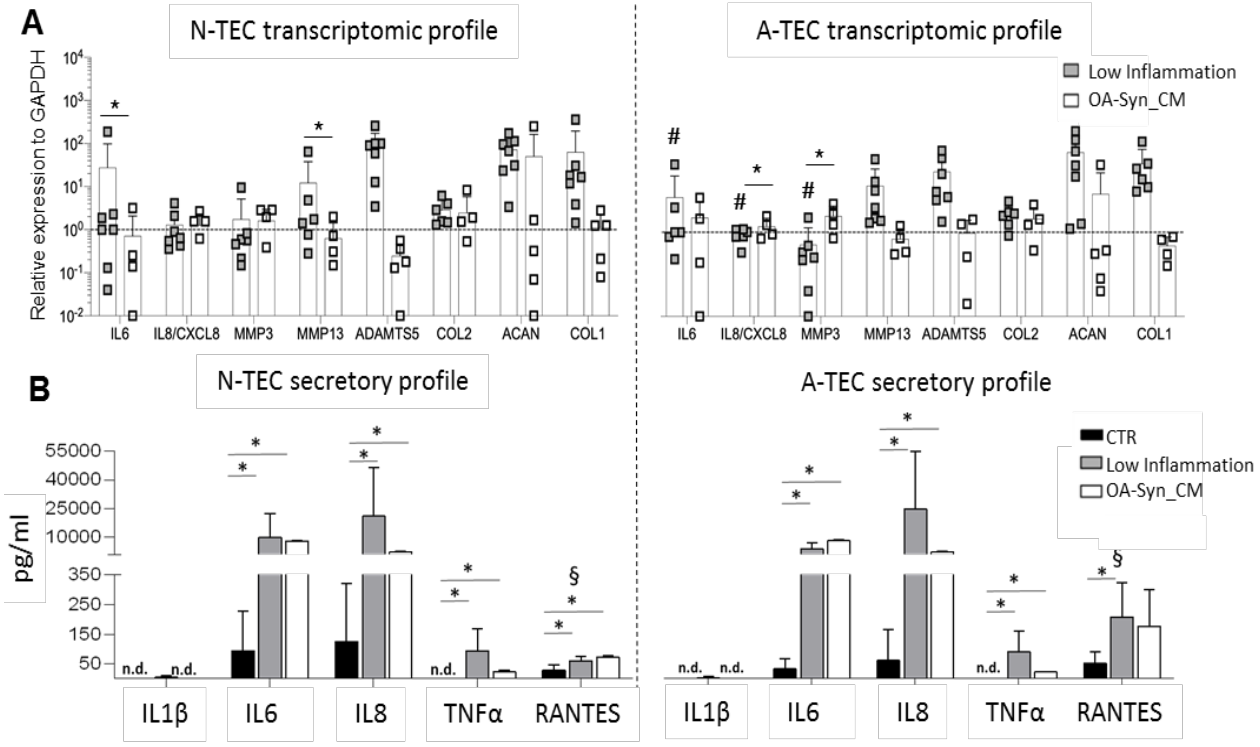

Figure 7. Transcriptomic and secretory profile of N-TEC and A-TEC exposed to exposed to *Low Inflammation* and conditioned medium of osteoarthritic synoviocytes (OA-Syn_CM). (A) RNA levels of inflammatory (IL6, IL8/CXCL8), catabolic (MMP3,-13, ADAMTS5) and cartilaginous factors (COL2,-1, ACAN) on nasal and articular chondrocytes from engineered tissues , after 7days of exposes to selected OA-conditions. Dashed line indicates expression of tissues in control condition (CTR). # Indicates significant differences versus CTR and * between conditions, for the same cell type and time point. (B) Levels of selected secreted factors by N-TEC and A-TEC at d7 (n = 4 matched donors). Values < 0.1 pg/ml were considered under the limit of detection (n.d. = not detected). * indicates significant changes versus CTR and $ between N-TEC and A-TEC at same time point and condition. Differences assessed by Mann-Whitney-U analyses. *P* <0.05 considered significant.

All this data indicates, that despite of the up-regulation of different inflammatory/catabolic factors upon the treatment with conditions mimicking more physiologically the inflammatory component of OA, N-TEC can better preserve their cartilaginous quality than N-TEC. Results also evidenced that the cellular phenotype of NC in terms of their gene expression and secretory profile, can be less affected than the phenotype of AC when stimulated by an inflammatory environment.

Table 5. Secretome of N-TEC and A-TEC upon exposure to *Low Inflammation* and medium conditioned by OA-Synovial cells (OA-Syn_CM). Cytokine concentrations (in pg/ml) measured in the supernatants of nasal or articular chondrocyte-based tissues (N-TEC/A-TEC) when cultured in basal medium (CTR) or in the OA-simulating conditions *Low Inflammation* and OA-Syn_CM. Values represented as the mean ±SD of n = 4 AC/NC matched donors. n.d. denotes not detected levels or values < 0.1. * indicates significant difference from CTR at the corresponding condition and $ significant difference between N-TEC and A-TEC at same condition. Differences assess by Mann-Whitney-U analyses. $P < 0.05$ considered significant.

	N-TEC			A-TECC		
	CTR	+Low Inflammation	+OA-Syn_CM	CTR	+Low Inflammation	+OA-Syn_CM
MIP1α/CCL3	n.d.	308.8 ±175.6*	385.7 ±27.3*	n.d.	341.5 ±110.8*	392.6 ±37.2*
MIP1β/CCL4	42.6 ±70.5	550.6 ±157.2*	811.2 ±61.9*	37.6 ±79.8	516.6 ±233.6*	803.1 ±52.7*
MCP1/CCL2	1244.4 ±662.7	12235.1 ±10318.7*	15934.0 ±3135.4*	876.0 ±964.4	13312.9 ±11768.5*	14734.6 ±2371.7*
IP10/CXCL10	0.7 ±2.0$	24.2 ±19.6*	66.3 ±4.7*	6.3 ±7.1$	94.6 ±33.9*	73.5 ±6.9*
GCSF	n.d.	136.9 ±273.9	n.d.	n.d.	29.5 ±34.7	n.d.
Eotaxin/CCL11	n.d.	n.d.	n.d.	n.d.	8.8 ±17.7	n.d.
IL1β	n.d.	4.7 ±6.5	n.d.	n.d.	3.1 ±3.59	n.d.
IL4	107.9 ±61.0	419.1 ±85.4*	472.3 ±20.6*	65.0 ±82.4	393.9 ±152.2*	486.3 ±18.3*
IL6	94.5 ±113.7	9985.0 ±12243.7*	7864.7 ±481.6*	32.6 ±33.6	3743.7 ±3053.4*	8019.9 ±535.7*
IL7	0.1 ±0.2	15.8 ±8.3*	22.0 ±1.7*	0.1 ±0.3	15.2 ±11.6*	22.1 ±0.9*
IL8/CXCL8	125.8 ±196.4	20918.6 ±25526.5*	2028.0 ±180.7*	61.7 ±103.6	24684.2 ±30265.7*	2061.6 ±164.7*
IL10	n.d.	2.1 ±1.5	2.9 ±0.5*	n.d.	2.3 ±2.7	4.4 ±1.0*
IL12	n.d.	157.6 ±99.6*	212.5 ±28.7*	n.d.	133.3 ±145.0	211.8 ±21.9*
IL13	n.d.	442.4 ±391.8	795.9 ±76.1*	n.d.	496.5 ±573.6	843.3 ±108.7*
IL17	n.d.	5.0 ±5.2*	5.8 ±1.6*	n.d.	4.9 ±5.66	6.8 ±2.8*
IL1RA	10.1 ±18.7	263.4 ±116.5*	952.1 ±116.1*	8.7 ±18.8	245.1 ±149.4*	964.6 ±140.4*
TNFα	n.d.	95.1 ±73.7*	23.1 ±2.9*	n.d.	91.1 ±69.0*	22.1 ±1.6*
IFNγ	n.d.	25.5 ±30.1	39.7 ±28.8	n.d.	33.4 ±38.6	48.3 ±20.6*
RANTES/CCL5	28.8 ±17.8	60.4 ±15.1*	73.1 ±5.4*	52.1 ±40.2	208.9 ±115.0*$	177.8 ±123.5
MMP3	35301.3±28555.2	64304.8 ±33620.0	30817.6 ±16311.4	21291.7 ±21093.4	97224.8 ±42922.2	17367.9 ±2660.9
MMP13	n.d.	161.5 ±323.0	n.d.	104.4 ±159.3	217.4 ±343.0	n.d.

4.1.3 Discussion

Results described in this section regarding the responses of nasal chondrocyte-based engineered constructs (N-TEC) to different OA-inflammatory conditions, evidenced the capacity of these tissues to preserve the cartilaginous matrix when exposed to inflammatory cues associated with an OA-environment. In particular, the histological quality of N-TEC was better preserved, as compare to A-TEC, regarding the content of typical ECM molecules such as GAG and type II collagen (Fig 4C, 6C). The superior capacity of N-TEC to withstand such conditions, was further supported by the findings of a non-affected expression profile upon exposure to the different assessed conditions, whereas AC-based tissues had higher levels of inflammatory and catabolic genes such as *IL6* and *MMP13,* in comparison to N-TEC (Fig. 5B) and CTR condition (Fig. 7A).

In additional analysis, detection of such markers at protein level evidenced that both N-TEC and A-TEC undergo a similar modulation on their secretory profiles upon inflammation. The only differentially modulated factor was RANTES, secreted with a significantly higher concentration by A-TEC, as compared N-TEC, in the condition of *Low Inflammation.* Thus, favoring the indications of better performance for NC-engineered tissues.

Overall, the here generated data coincides with previous studies comparing the chondrogenic capacity of nasal and articular chondrocytes, regarding the higher content of GAG and type II collagen in NC-based tissues that in A-TEC at control conditions. In those studies, GAG loss has been also reported as result of the short IL1β stimulus (3d) or a prolonged exposure (up to 21d) to the same cytokine cocktail hence used (IL1β, IL6, and TNFα), but in a medium with glucose deprivation and under hypoxic conditions *(154, 242)*. Conversely, in this thesis no significant up-regulation of catabolic factors such as MMP13 was observed neither in N-TEC nor in A-TEC, as reported when more 'acute' inflammatory conditions were used (i.e., high dose, short exposure to IL1β). Such distinctive response could be presumably attributed to the differential role that diverse

levels of inflammation and exposure times might play in the promotion of a catabolic environment *(164)*.

As a limitation for the interpretation of the current data, the GAG measurement - as single parameter - was found non-conclusive for assessing the responses of engineered tissues to the conditions here tested. This, considering that although the majority of inflammatory conditions caused a decreased GAG content in both N-TEC and A-TEC, the same effect was observed with the medium used as control (CTR). Such observations prevented a clean discrimination of the effect caused by the used factors (cytokines or CM), among other potential detrimental conditions such as the presence of serum *(244)* and the absence of TGF-β. The supplementation with serum could not be avoided due to its relevance for the culture of OA-Syn (from which CM was derived), and the TGF-β was not included in order to have conditions better representing those used for the clinical manufacturing of N-TEC *(157)*.

Complementary analysis for better understanding of the differential nature between nasal and articular chondrocytes, as well as the superior potential of NC for clinical applications, might include a comparative transcriptomic analysis of both cell types (ongoing in our group). This will support the elucidation of innate cell features, such as differential expression levels of inflammatory (Data included in submitted manuscript), anabolic and catabolic factors (to be further investigated). In order to better characterize the responses of N-TEC and A-TEC to the implemented OA simulating conditions, as well as to understand more broadly the mechanisms underlying disease progression or recovery, the study of cellular processes - other than the metabolic/inflammatory ones- could be of critical relevance. Such additional outlooks could be focused on the roles of senescence, autophagy, apoptosis and oxidative stresses *(245–247)*.

4.2 Aim II: Modulatory effects of N-TEC on the transcriptomic and secretory profiles of osteoarthritic cells and tissues

4.2.1 Introduction

In the osteoarthritic knee, the increased concentrations of circulating inflammatory and catabolic molecules is a well-established feature of the disease, when compared to healthy condition *(174)*. Considered a pathology of the joint as a whole *(162)*, those factors can be secreted by the chondrocytes themselves *(248, 249)* and/or by the cells present in the adjacent tissues such as the synovial membrane *(250, 251)* and subchondral bone *(175, 218)*. Consequently, after determining the effects that those mediators exert on nasal chondrocytes - in the form of engineered cartilage - (*Aim I*), it was imperative to evaluate the modulatory effects that N-TEC could have in the production of the mentioned molecules, in order to understand the implications of a multidirectional interaction between the different cell/tissue types within the joint.

With that intention, the aim was to determine the effects of N-TEC secreted factors on the phenotype of osteoarthritic articular chondrocytes (OA-AC), synoviocytes (OA-Syn) and osteochodral tissue explants (OA-OC). Pro-inflammatory and catabolic profiles of the chosen models have been previously established through immunodetection and cytokine profiling for OA-Syn *(171, 252)*, as well as with protein quantification or gene expression analysis for OA-OC explants or osteoblasts (OB) isolated from an OA joint *(175, 193)*. In the case of OA-AC, their phenotype have been characterized as unstable in terms of de-differentiation, reduced expression of anabolic molecules, and increased secretion of catabolic and inflammatory factors OA-OA *(174, 249, 253)*.

Attenuation of the described OA-related phenotypes have been also reported in OA-Syn by secreted factors from adipose-derived stromal cells *(252)*, in OA-OC by inhibition of the TGF-β

pathway *(193)* and in OA-AC by stimulation with a chemical agent, in a scaffold-based culture system *(219)*.

Although the capacity of nasal chondrocytes to withstand degenerative-simulating conditions have been demonstrated in different contexts (*Aim I*, *(242)*), their potential anti-inflammatory/anti-catabolic properties have not been yet investigated. This chapter constitutes the first approach in that direction - in the frame of osteoarthritis -, and summarizes the findings of the afore described experimental models.

4.2.2 Results

4.2.2.1 Modulation of the inflammatory/catabolic profile of osteoarthritic articular chondrocytes by nasal chondrocytes-secreted factors

Addressing whether nasal chondrocytes - as the therapeutic agent explored in this manuscript - could have an anti-inflammatory/catabolic effect on OA-AC; 3D aggregates of these cells were exposed to media conditioned by nasal chondrocytes (N-TEC_CM), at micro and macroscales, (*section 7.3.1.3*) or to CTR. The conditioned medium from healthy articular chondrocytes (A-TEC_CM) was used (i) as reference cell from the targeted tissue (i.e., articular cartilage) and (ii) to assess whether effects were specific to the nasal origin of the cells, or a general chondrocyte-related feature. Modulatory effects of the different conditions were then determined by qRT-PCR, immunofluorescence and/or protein quantification according to the specific model.

4.2.2.1.1 Optimization of experimental conditions for the exposure of OA-AC to conditioned media

It was previously shown that microaggregates (of about 100cells/unit) cultured in a microfluidic device have a more uniform and reproducible response to continuously perfused stimuli *(254)*. In the system here described, the stimulus consisted of the perfusion of N-TEC or A-TEC derived

conditioned media. Parallel studies indicated that the culture with 80% of N-TEC_CM during 7 days post-differentiation was capable of maintaining the chondrogenic ECM, as assessed by immune-detection of type II collagen, ans sufficient to reduce the production of catabolic and factors inflammatory by OA-AC, such as the MMP13 at protein level (Fig. 8A) and *ADAMTS5, MMP13* and *IL8* at mRNA level (Fig. 8B) *(255)*. For this reason, stimulation for 7d and 80% CM were the chosen conditions for the entire setup.

4.2.2.1.2 N-TEC secreted factors down regulate the production of inflammatory and catabolic factors in OA-AC

To evaluate the responses of OA-AC to the medium conditioned by N-TEC and A-TEC (N-TEC_CM/A-TEC_CM), NC and AC were isolated, expanded, chondrogenically differentiated and used to produce the conditioned medium. In parallel, AC form OA donors (OA-AC) were also isolated and used to form 3D aggregates that were further exposed to CTR or to conditioned media in two different culture systems; at micro and macro scales (Fig. 9A).

4.2.2.1.2.1 Microscale

After exposure to experimental conditions, gene expression analysis of OA-AC exposed to N-TEC_CM had a significant reduction in transcription levels of the inflammatory cytokine *IL8* and the catabolic factor *MMP13* as compared to CTR (basic culture medium), with the same trend followed by the aggrecanase enzyme *ADAMTS5* (P =0.0609). This reduction did not occur in the presence of A-TEC_CM (Fig. 9B). The anti-catabolic effect on MMP13 reduction was further ratified at protein level, with a low detection of the enzyme in N-TEC_CM treated microaggregates, as compared to CTR or A-TEC_CM treated OA-AC (Fig. 9C). Due to the scale, no material for additional analysis could be harvested from this culture system.

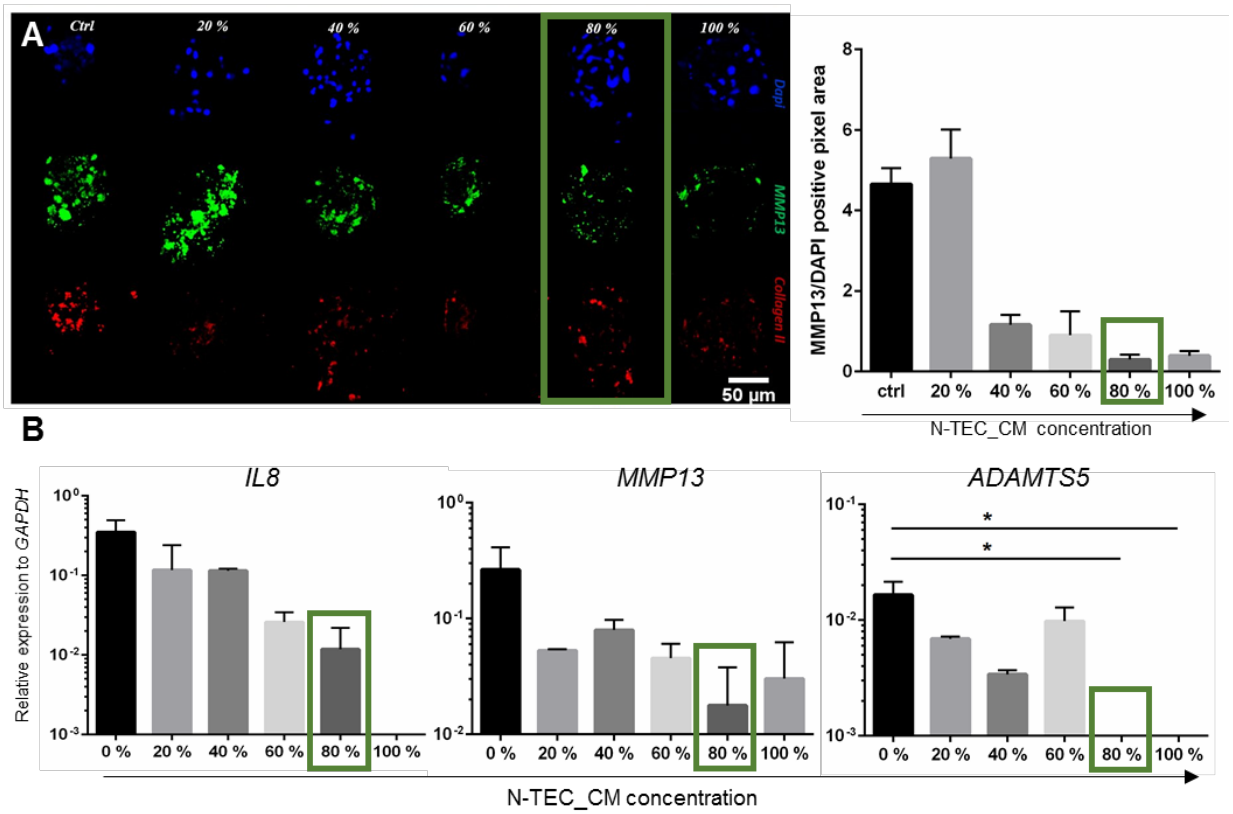

Figure 8. Modulation of catabolic, anabolic and inflammatory markers in OA-AC by concentrations of N-TEC_CM. (**A**) Immuno-fluorescent detection of the catabolic marker MMP13 and the anabolic type II collagen, in 3D aggregates of osteoarthritic articular chondrocytes (OA-AC) after exposure to different concentrations of N-TEC_CM - medium conditioned by tissue engineered cartilage generated with nasal chondrocytes – (left). Quantification of the staining for MMP13 presented on the right. (**B**) OA-AC expression levels for the pro-inflammatory marker *IL8* and the catabolic *MMP13* and *ADAMTS5,* after N-TEC_CM treatment. Green square indicates the concentration of 80% CM, in which levels of catabolic and inflammatory factors were lower while still preserving some type II collagen.

4.2.2.1.2.2 Macroscale

In order to perform a broader characterization than in the micro-system, a standard macroaggregate culture ($2.5x10^5$ cells/unit) was implemented *(256)*. In this latest setup, and similarly to what was observed in the microaggregates, the culture of OA-AC with N-TEC secreted factors caused a significant reduction in the mRNA levels of *MMP13* and *ADAMTS5*, while *IL8* levels remained at the same level than CTR treated macroaggregates. Instead, culture of OA-AC with A-TEC_CM significantly increased the production of *IL8* transcripts. In addition, the modulation of *IL6* mRNA

was assessed in this model, being not significantly regulated by any of the tested conditions (Fig. 9D). Detection of MMP13 by immunofluorescence demonstrated the absence of this molecule in the OA-AC treated with N-TEC_CM, whereas it was present in the macroaggregates exposed to both CTR and A-TEC_CM (Fig 9E).

To quantify the responses at protein level, factors secreted by OA-AC when exposed to N-TEC_CM or A-TEC_CM were *measured* in the culture supernatants and compared to *expected* concentrations (calculated as the sum of the amount secreted by OA-AC at CTR condition plus the amount measured in the N-TEC_CM or A-TEC_CM). This assessment revealed that neither nasal or healthy articular chondrocytes conditioned media could modulate the secretion of the majority of the factors such as MIP1α, MIP1β, IP10, IL1β, IL7, IL10, IL13, IL1Ra, TNFα, RANTES, MMP3 and MMP13. Among the whole panel assessed, the secretion of IL12 was suppressed by N-TEC_CM, IP10 by A-TEC_CM and INF-γ by both N-TEC/A-TEC_CM. Up-regulation was only observed in the presence of A-TEC_CM for the cytokines MCP1, IL4, IL6 and IL8 (Table 6). The observed modulatory effects on factors classically studied by their relevance in OA (IL1β, IL6, IL8, TNFα and RANTES) are illustrated in Fig. 9F.

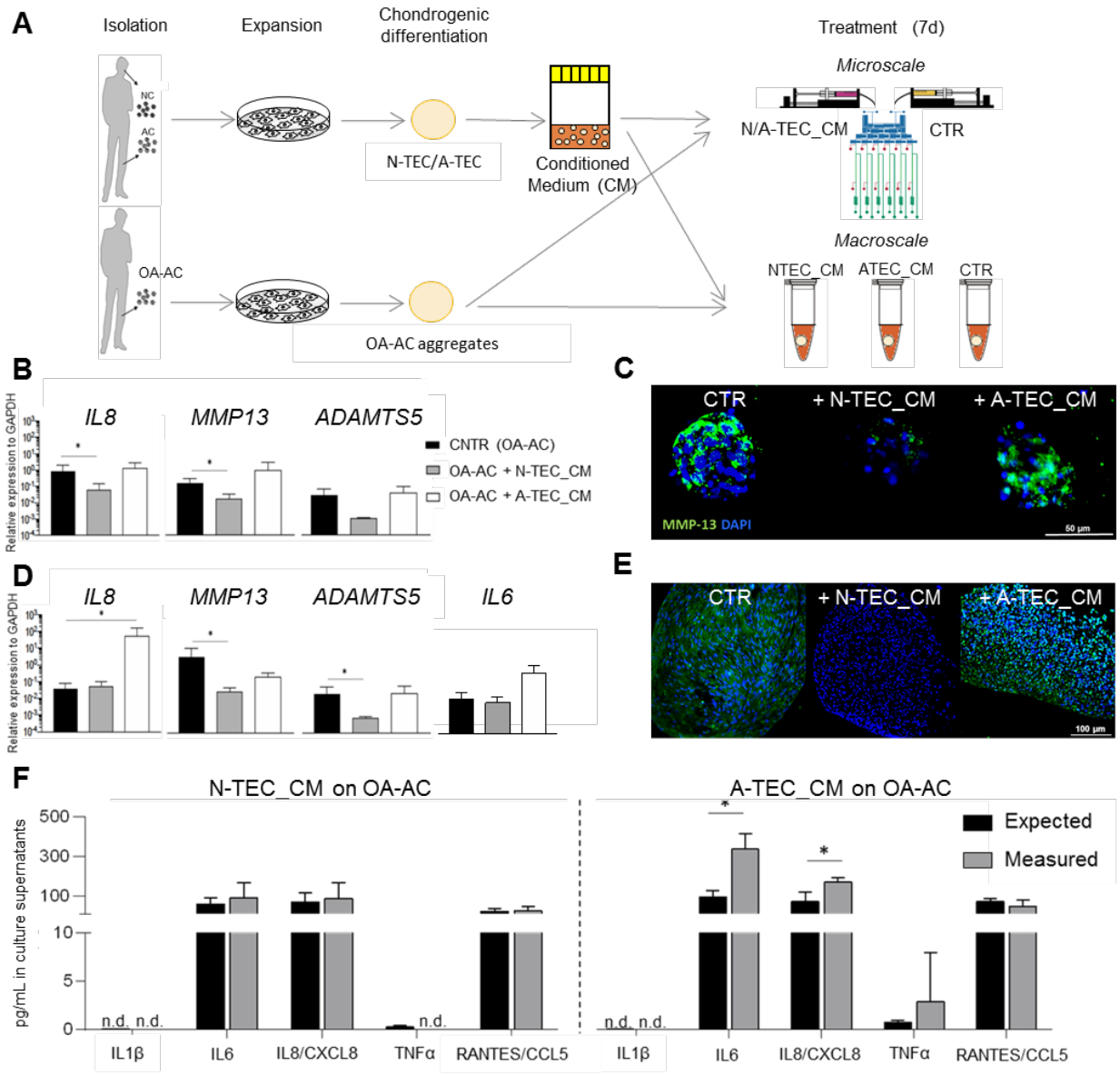

Figure 9. Modulatory effects of N-TEC and A-TEC secreted factors on the phenotype of osteoarthritic articular chondrocytes (OA-AC). (**A**) Schematic representation of experimental design. (**B**) Expression levels of microaggregates generated with OA-AC (n = 2 donors) for selected factors, following exposure to CTR or to medium conditioned by N-TEC (N-TEC_CM) and A-TEC (A-TEC_CM) (both pooled from n=5 donors). (**C**) Immunofluorescent detection of MMP13 in micro-aggregates. (**D**) Expression levels of OA-AC macroaggregates (n= 2 donors) for selected factors, following exposure to CTR and N-TEC/A-TEC_CM. (**E**) Immunofluorescent detection of MMP13 in OA-AC macroaggregates. (**F**) *Expected* (sum of values for OA-AC (n = 3 donors) in CTR plus amounts quantified in N-TEC_CM or A-TEC_CM) and *Measured* (values of OA-AC cultured in N-TEC_CM or A-TEC_CM) concentrations of selected factors quantified in culture supernatants. n.d. denotes not detected levels or values < 0.1. Differences assess by Mann-Whitney-U analyses. $P < 0.05$ considered significant.

Table 6. Secretome of OA-AC upon exposure to N-TEC and A-TEC secreted factors. Cytokine concentrations (in pg/ml) measured in the supernatants of osteoarthritic chondrocytes (OA-AC) when cultured in CTR or in N-TEC or A-TEC conditioned medium (N-TEC/A-TEC_CM). Values represented as the mean ±SD of n = 3 OA-AC donors and n = 1 CM (pooled from 5 donors). n.d. denotes not detected levels or values < 0.1. * indicates a significant difference between measured and expected values, assessed by Mann-Whitney U test. Unilateral $P \leq 0.05$ considered significant.

	Single cells			OA-AC+N-TEC_CM		OA-AC+A-TEC_CM	
	N-TEC_CM	A-TEC_CM	OA-AC	Expected	Measured	Expected	Measured
MIP1α/CCL3	n.d.	n.d.	n.d.	n.d.	107.1 ± 95.5	n.d.	73.8 ± 127.8
MIP1β/CCL4	n.d.	n.d.	n.d.	n.d.	53.6 ± 64.0	n.d.	130.9 ± 196.2
MCP-1/CCL2	136.6 ± 102.9	78.9 ± 44.8	728.7 ± 633.3	865.3 ± 633.3	1296.9 ± 1166.0	807.6 ± 633.3	2760.4 ± 2242.1*
IP10/CXCL10	n.d.	1.6 ± 3.6	n.d.	n.d.	n.d.	1.6 ± 3.6	n.d.*
GCSF	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Eotaxin/CCL11	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
IL1β	0.1 ± 0.1	0.1 ± 0.1	n.d.	0.1 ± 0.1	n.d.*	0.1 ± 0.1	n.d.*
IL4	0.1 ± 0.2	0.1 ± 0.1	67.0 ± 58.2	67.1 ± 58.2	107.9 ± 95.7	67.1 ± 58.2	193.7 ± 113.4*
IL6	32.0 ± 33.5	69.6 ± 53.3	27.8 ± 32.8	59.8 ± 32.8	91.0 ± 78.1	97.4 ± 32.8	337.0 ± 77.8*
IL7	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	2.1 ± 3.6
IL8/CXCL8	9.6 ± 7.5	11.5 ± 8.7	60.8 ± 47.9	70.4 ± 47.9	88.3 ± 78.8	72.3 ± 47.9	171.8 ± 22.3*
IL10	0.1 ± 0.2	n.d.	n.d.	0.1 ± 0.2	n.d.*	n.d.	n.d.
IL12	20.5 ± 13.0	19.0 ± 12.0	n.d.	20.5 ± 13.0	n.d.*	19.0 ± 12.0	10.9 ± 18.9
IL13	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
IL17	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
IL1Ra	n.d.	0.2 ± 0.5	n.d.	n.d.	12.4 ± 21.5	0.2 ± 0.5	43.5 ± 75.3
TNFα	0.3 ± 0.6	0.8 ± 1.1	n.d.	0.3 ± 0.6	n.d.*	0.8 ± 1.1	2.9 ± 5.0
IFNY	3.1 ± 4.3	4.1 ± 6.4	n.d.	3.1 ± 4.3	n.d.*	4.1 ± 6.4	n.d.*
RANTES/CCL5	n.d.	48.0 ± 44.2	24.1 ± 14.2	24.1 ± 14.2	25.8 ± 23.4	72.2 ± 14.2	49.8 ± 30.6
MMP3	n.d.	n.d.	22407.4 ± 14682.5	22407.4 ± 14682.5	20144.4 ± 15626.0	22407.4 ± 14682.5	32826.8 ± 10275.6
MMP13	62.4 ± 27.9	4981.4 ± 5034.9	n.d.	62.4 ± 27.9	37.0 ± 64.1	4981.4 ± 5034.9	187.3 ± 324.5
ADAMTS5	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

4.2.2.2 Modulation of the inflammatory profile of osteoarthritic synovial cells by nasal chondrocytes secreted factors

Synovial cells are the typically reported source of inflammatory factors in an OA joint *(160)*. Therefore, after assessing the modulatory effects of N-TEC_CM on OA-AC, it was important to determine the potential of N-TEC secreted factors to down-regulate the inflammatory phenotype of this cell type. For this purpose, cells from the synovial tissue of osteoarthritic joints (OA-Syn) were isolated and cultured in the presence of N-TEC_CM, A-TEC_CM or CTR (*section 7.3.1.5*) (Fig. 10A). Regulatory effects of these conditions were then assessed by quantification of the cytokines in the resulting culture supernatants, as well as by qRT-PCR.

4.2.2.2.1 Optimization of experimental conditions for the exposure of OA-Syn to conditioned media

Comparison of the secretory profile of OA-Syn cultured during 2 or 7 days showed that all the inflammatory related molecules measured (MIP1α, MIP1β, MCP1, IP10, IL1β, IL6, IL7, IL8, IL10, IL12, IL1RA, TNFα, INFγ and RANTES) were already secreted into the supernatant by day 2. The prolongation of the culture conditions (up to 7 days) caused either a drop or a suppression of the majority of the factors, being MCP1and IL6 the only factors whose concentration slightly increased at the latest measured time (Fig. 10B). For this reason, the duration for exposing OA-Syn to the conditioned media or CTR was set at 2 days.

In order to determine the appropriate concentration of CM effectively modulating the secretory profile of OA-Syn, this OA cell type was exposed to 100, 50 or 25% (v/v in CTR medium) of N-TEC_CM or CTR (0% CM), to then measure the concentrations of the targeted cytokines (same panel than for the timing comparison). In these conditions, the concentrations of 100 and 50% reduced the secreted levels of MIP1α, MIP1β, MCP1, IL1β, IL6, IL7, IL8, INFγ and RANTES in OA-Syn, as compared to the concentration of 25% and the basal secreted levels at 0%. Those same higher concentrations suppressed the production of IP10, IL1RA and TNFα. Only for IL7 and IL10 - out of the fourteen factors measured - the concentration levels were lower at the condition of 25% than it at least one of the other concentrations of N-TEC_CM. No relevant differences in the effect were detected between 100% and 50% (Fig. 10C), therefore CM was diluted 1:1 with CTR medium for all forthcoming experiments.

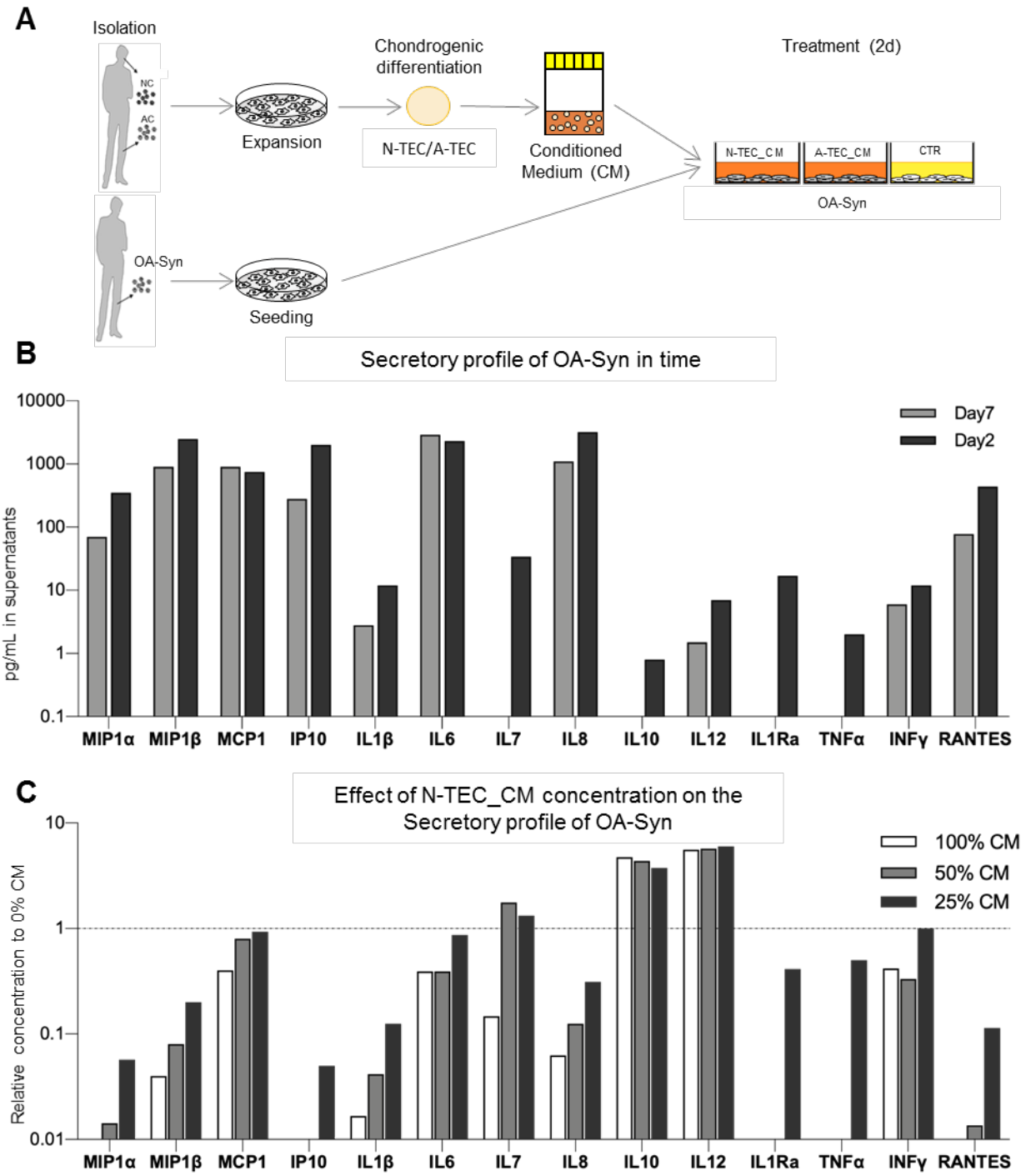

Figure 10. Secretory profile of OA-Syn and modulation by culture time and exposure to N-TEC_CM. (A) Schematic representation of experimental design. (B) Secreted concentrations of selected factors by osteoarthritic synovial cells (OA-Syn) in different culture times and (C) when exposed to different concentrations of medium conditioned by N-TEC (N-TEC_CM). Values corresponding to measurements in 1 (out of 2) used OA-Syn donors. Dashed line indicates the secreted values by OA-Syn in CTR (0% CM).

4.2.2.2.2 N-TEC secreted factors down regulate the production of inflammatory and catabolic factors in OA-Syn

Once experimental conditions (2days of exposure to 50% CM) were established, factors secreted by OA-Syn when cultured with N-TEC_CM or A-TEC_CM were *measured* in the supernatants and compared to *expected* concentrations (calculated as the sum of the amount secreted by OA-Syn at CTR plus the amount measured in the N-TEC_CM or A-TEC_CM). Quantification revealed that the majority of the factors were similarly modulated in the presence of both N-TEC and A-TEC_CM, by significantly decreasing levels of MIP1α, MIP1β, MCP1, IP10, IL1β, IL8, IL17 IL1RA and RANTES, while increasing production of IL10, IL12 and IL13. Differentially, only the culture with N-TEC_CM caused a significant downregulation of IL6 and TNFα (P= 0.025 and P= 0.028 respectively). Modulation of selected factors (IL1β, IL6, IL8, TNFα, RANTES) is illustrated in Fig. 11A, and the measured values for the extended panel presented in Table 7. In the same setup, gene expression analysis confirmed the significant reduction of IL8 levels only for the N-TEC_CM treated OA-Syn. Nasal chondrocytes secreted factors additionally downregulated the expression of MMP13 and ADAMTS5, as compared to control condition and in line with the findings for OA_AC. This modulation did not occur in the presence of A-TEC_CM (Fig. 11B).

4.2.2.3 Modulation of the secretory profile of osteoarthritic osteochondral explants by nasal chondrocyte secreted factors

In addition to OA-AC and OA-Syn, cells present in the subchondral bone of an OA joint also contribute to the increased circulating concentrations of inflammatory and catabolic mediators *(161)*. For this reason, it was of complementary interest to evaluate the effects of N-TEC released factors on the secretory profile of osteoarthritic-osteochondral explants (OA-OC). Consequently, similarly to the setups implemented for OA-AC/Syn, in this experimental setting pieces of OA-OC tissues were isolated and cultured in the presence N-TEC_CM, A-TEC_CM or CTR (Fig. 12A).

Modulatory effects on selected factors were assessed by histological characterization and protein quantification. Duration of the exposures was set at 7days according to previous studies where these ex-vivo osteochondral explants have been used *(193)*. Concentration for chondrocyte conditioned media was chosen accordingly to the one we found effective for modulating the OA-AC profile (at 80%).

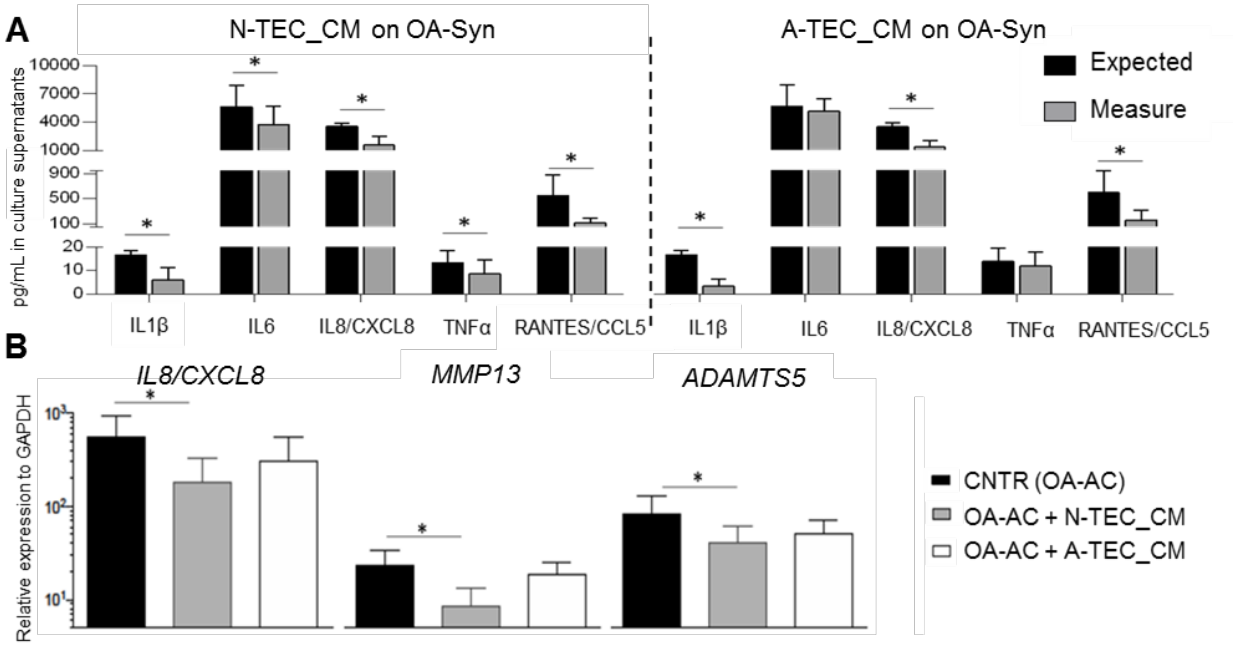

Figure 11. Modulatory effects of N-TEC and A-TEC secreted factors on the phenotype of osteoarthritic synovial cells (OA-Syn). (A) Concentrations of selected factors secreted by OA-Syn (n = 6 donors) after exposure to CTR or to medium conditioned by N-TEC or A-TEC (A-TEC_CM) (n = 4 donors each). Values represented as the mean ±SD of Expected (sum of values for OA-syn in CTR plus amounts quantified in N-TEC_CM or A-TEC_CM) and Measured (values of OA-Syn cultured in N-TEC_CM or A-TEC_CM) concentrations quantified in culture supernatants. (B) Expression levels of selected genes by OA-Syn for the different experimental conditions. 12 experimental replicates measured per condition. n.d. denotes not detected levels or values < 0.1. * indicates a significant difference between measured and expected values, assessed by Mann-Whitney U test. Unilateral $P \leq 0.05$ considered significant.

Table 7. Secretome of OA-Syn upon exposure to N-TEC and A-TEC secreted factors. Cytokine concentrations (in pg/ml) measured in the supernatants of osteoarthritic synovial cells (OA-Syn) when cultured in CTR or in N-TEC or A-TEC conditioned medium (N-TEC/A-TEC_CM). Values represented as the mean ±SD of 12 experimental replicates for n = 3 Syn donors, randomly combined with n = 4 CM donors. n.d. denotes not detected levels or values < 0.1. * indicates a significant difference between measured and expected values, assessed by Mann-Whitney U test. Unilateral $P < 0.05$ considered significant.

	Single cells			OA-Syn+N-TEC_CM		OA-Syn+A-TEC_CM	
	N-TEC_CM	A-TEC_CM	OA-Syn	Expected	Measured	Expected	Measured
MIP1α/CCL3	n.d	n.d	433.1 ± 60.3	433.1 ± 60.3	107.7 ± 137.1*	433.1 ± 60.3	50.4 ± 60.4*
MIP1β/CCL4	n.d	n.d	2859.6 ± 287.7	2859.6 ± 287.7	1413.9 ± 644.3*	2859.6 ± 287.7	1308.7 ± 529.1*
MCP-1/CCL2	170.8 ± 79.7	98.6 ± 8.9	662.0 ± 126.8	786.9 ± 139.8	569.5 ± 99.6*	714.7 ± 105.9	528.5 ± 62.8*
IP10/CXCL10	n.d	2.0 ± 4.0	9626.4 ± 8600.6	9626.4 ± 8600.6	3358.4 ± 5260.0*	9628.5 ± 8602.0	2002.9 ± 2332.9*
GCSF	n.d	n.d	3.0 ± 1.4	3.0 ± 1.4	7.2 ± 7.2	3.0 ± 1.4	4.8 ± 5.6
Eotaxin/CCL11	n.d	n.d	23.6 ± 13.5	23.6 ± 13.5	42.8 ± 53.3	23.6 ± 13.5	34.4 ± 45.4
IL1β	0.1 ± 0.1	0.1 ± 0.1	15.8 ± 2.6	16.6 ± 2.0	5.9 ± 5.3*	16.6 ± 2.0	3.3 ± 3.1*
IL4	0.2 ± 0.2	0.1 ± 0.1	1.9 ± 1.1	2.3 ± 0.9	1.9 ± 0.76*	2.3 ± 0.9	1.7 ± 0.5*
IL6	40.0 ± 32.8	87.0 ± 42.0	5070.8 ± 2665.4	5629.4 ± 2262.4	3759.3 ± 1913.0*	5673.7 ± 2267.7	5141.4 ± 1292.2
IL7	n.d	n.d	16.9 ± 8.7	16.9 ± 8.7	15.9 ± 11.6	16.9 ± 8.7	14.2 ± 5.2
IL8/CXCL8	12.0 ± 6.0	15.4 ± 4.9	3538.6 ± 399.2	3532.9 ± 392.1	1554.7 ± 925.4*	3533.3 ± 391.7	1354.5 ± 667.3*
IL10	0.2 ± 0.2	n.d	2.9 ± 0.8	3.0 ± 0.7	7.7 ± 2.9*	2.9 ± 0.8	6.7 ± 2.6*
IL12	25.6 ± 7.2	23.7 ± 6.4	22.8 ± 14.2	51.1 ± 9.6	67.5 ± 11.2*	49.6 ± 11.3	76.4 ± 12.5*
IL13	n.d	n.d	0.5 ± 0.2	0.5 ± 0.2	1.0 ± 0.5*	0.5 ± 0.2	1.1 ± 0.5*
IL17	n.d	n.d	21.2 ± 10.3	21.2 ± 10.3	14.1 ± 8.6*	21.2 ± 10.3	11.0 ± 5.8*
IL1RA	n.d	n.d	109.5 ± 73.5	109.5 ± 73.5	49.0 ± 44.8*	109.5 ± 73.5	56.4 ± 54.2*
TNFα	0.3 ± 0.7	1.0 ± 1.1	11.1 ± 6.4	13.3 ± 5.3	8.5 ± 6.3*	13.9 ± 5.7	12.0 ± 5.8
IFNY	3.8 ± 4.6	5.2 ± 6.9	75.9 ± 48.7	93.6 ± 40.0	80.2 ± 38.9*	95.0 ± 41.2	76.6 ± 32.9
RANTES/CCL5	n.d	60.1 ± 40.5	548.2 ± 331.4	548.2 ± 331.4	114.6 ± 73.6*	601.3 ± 343.8	160.1 ± 157.3*

4.2.2.3.1 N-TEC secreted factors down regulate the production of inflammatory and catabolic factors in OA-OC tissues

After exposure to experimental conditions, secreted levels of MIP1α, MIP1β, GCSF, IL6, IL10, IL1Ra, TNFα, RANTES, MMP13, ADAMTS4 and ADAMTS5 were *measured* in the culture supernatants and compared to *expected* concentrations (calculated as the sum of the amount secreted by OA-AC at CTR condition plus the amount measured in the N-TEC or A-TEC_CM). Such quantification revealed that both conditioned media had similar effect on the secretome of OA-OC with no modulation on MIP1β, GCSF, IL6 and ADAMTS5, and a significant down regulation in levels of MIP1α, IL10, IL1Ra, TNFα, MMP3 and ADAMTS4. RANTES was the

only factor differentially affected by nasal or articular chondrocytes-derived conditioned medium, being significantly downregulated just in the presence of A-TEC_CM (Table 8).

No modulation on extracellular composition was evidence by immunohistochemical detection of GAG and type-II/I Collagens (Fig. 12B,C).

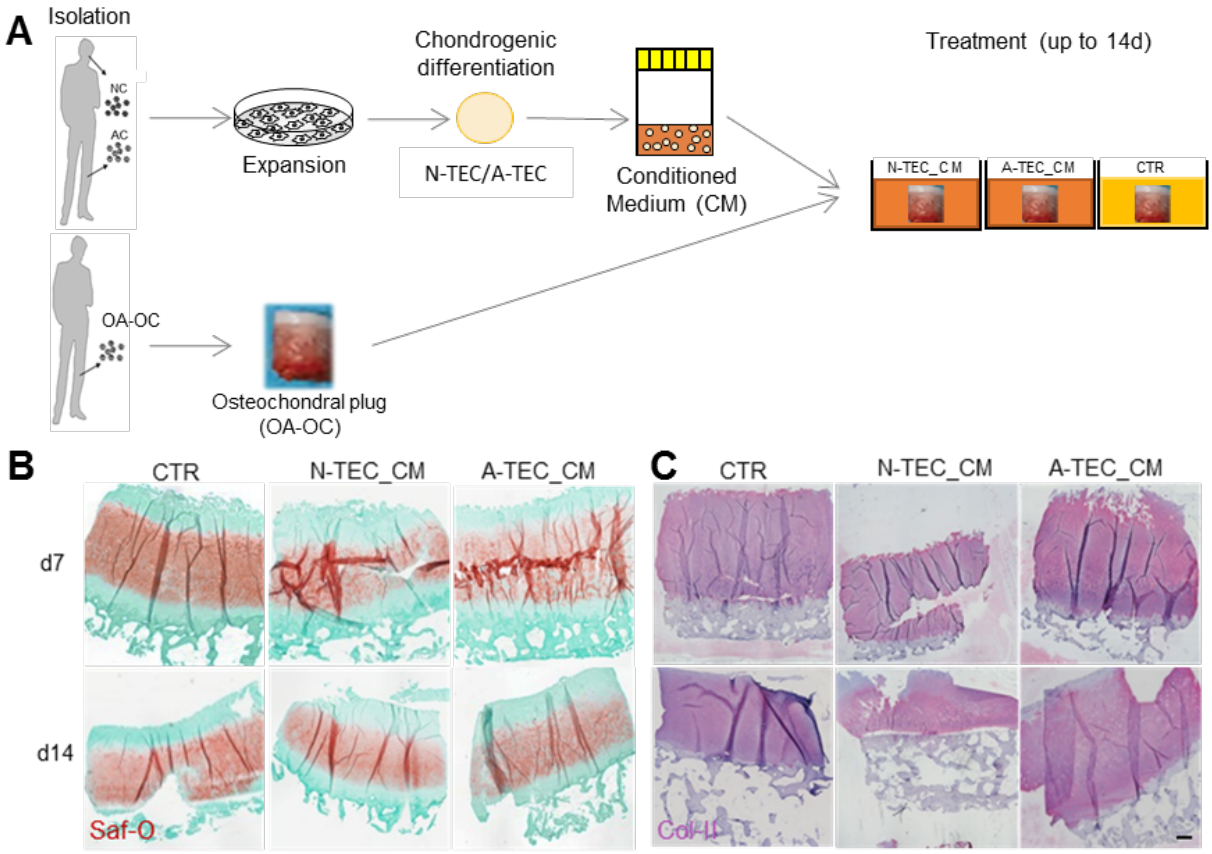

Figure 12. Modulatory effects of N-TEC and A-TEC secreted factors on the phenotype of osteoarthritic osteochondral plugs (OA-OC). (**A**) Schematic representation of experimental design. (**B**) Safranin-O staining and (**C**) type II collagen immunohistochemical detection, for OA-OC tissues (n = 2 donors) upon exposure to CTR or medium conditioned by N-TEC (N-TEC_CM) and A-TEC (A-TEC_CM) (n = 2 donors). Scale bar = 1000 μm.

Table 8. Secretome of OA-OC upon exposure to N-TEC and A-TEC secreted factors. Cytokine concentrations (in pg/ml for CM[§] and in pg/mg for OA-OC and combinations[¥]) measured in the supernatants of osteoarthritic osteochondral tissues (OA-OC) when cultured in CTR, N-TEC or A-TEC conditioned medium (N-TEC/A-TEC_CM). Values represented as the mean ±SD of 4 experimental replicates for n= 2 OA-OC donors combined with n= 2 CM donors. n.d. denotes not detected levels or values < 0.1. * indicates a significant difference between measured and expected values, assessed by Mann-Whitney U test. Unilateral $P \leq 0.05$ considered significant.

	Controls			OA-OC+N-TEC_CM[¥]		OA-OC+A-TEC_CM[¥]	
	NTEC-CM[§]	ATEC-CM[§]	OA-OC[¥]	Expected	Measured	Expected	Measured
MIP1α	n.d.	n.d.	15.0 ± 4.9	20.1 ± 8.1	18.8 ± 8.0*	19.5 ± 6.4	19.2 ± 6.0*
MIP1β	18.9 ± 1.1	26.7 ± 10.3	0.7 ± 0.2	1.2 ± 0.4	7.9 ± 13.1	1.3 ± 0.3	3.0 ± 3.8
GCSF	3.6 ± 0.3	5.1 ± 1.7	0.1 ± 0.0	0.2 ± 0.1	0.1 ± 0.1	0.2 ± 0.1	0.1 ± 0.0
IL6	40.0 ± 33.5	87.0 ± 53.3	90.3 ± 34.9	123.0 ± 41.9	127.2 ± 67.5	120.5 ± 32.3	111.8 ± 50.2
IL10	22.9 ± 4.1	30.6 ± 7.7	0.6 ± 0.2	1.1 ± 0.4	0.7 ± 0.3*	1.1 ± 0.4	0.7 ± 0.2*
IL1Ra	242.0 ± 14.3	320.3 ± 51.1	5.4 ± 1.7	10.3 ± 4.1	7.2 ± 3.9*	11.0 ± 3.6	6.6 ± 1.9*
TNFα	9.2 ± 1.6	42.6 ± 20.3	0.4 ± 0.1	0.7 ± 0.3	0.6 ± 0.4*	1.1 ± 0.4	0.6 ± 0.2*
RANTES	n.d.	0.0 ± 0.0	25.3 ± 21.4	35.0 ± 23.1	28.0 ± 45.9	33.7 ± 20.6	10.9 ± 15.0*
MMP3	22378.7 ± 3487.7	46495.3 ± 31087.0	1770.2 ± 402.5	2640.3 ± 876.1	1592.5 ± 726.0*	2866.9 ± 736.2	1736.7 ± 518.4*
ADAMTS4	6.0 ± 9.5	7.5 ± 11.2	0.3 ± 0.2	0.6 ± 0.2	0.1 ± 0.1*	0.7 ± 0.2	0.2 ± 0.1*
ADAMTS5	n.d.	n.d.	3.2 ± 3.7	5.4 ± 2.2	5.3 ± 5.9	6.3 ± 3.7	6.3 ± 5.4

4.2.3 Discussion

The findings described above demonstrate that factors released by engineered cartilaginous tissues generated with adult nasal chondrocytes (N-TEC), can attenuate the inflammatory and catabolic profile of osteoarthritic (OA) cells/tissues in a target-specific manner, inducing differential responses on each of the experimental setups.

Although the main interest of this investigation resides on the potential use of N-TEC for the treatment of OA lesions, the secreted factors of A-TEC were studied in addition, resulting on a similar modulation of the targeted profiles in most of the cases. However differential modulation of the more typically OA-related factors was evidenced as positive only for the N-TEC_CM, regarding exclusive significant downregulation of *IL8, MMP13* and *ADAMTS5* gene expression levels in both OA-AC and OA-Syn, as well as a significantly reduced secreted concentrations of

IL6 and TNFα in OA-Syn (Fig. 9,11). This differential effect among N-TEC and A-TEC secreted factors, was additionally supported by the increased production of pro-inflammatory factors by OA-AC, exclusively caused by the presence of A-TEC_CM, as observed for *IL8* at mRNA level (Fig. 9) and different other cytokines at protein level - including IL8 and IL6 - (Table 6).

It is presumed that the anti-inflammatory/catabolic properties of showed by N-TEC secreted factors, in combination with the chondrogenic matrix maintenance under degenerative-simulating conditions previously demonstrated (*section 4.1*, *(242)*), may be beneficial for the long-term performance of the graft. However, further studies are needed in order to understand the contribution of these two features to a potential delay in the progression of the disease, a structural amelioration of the joint, or a presumable restoration of cartilage homeostasis. Considering these last two parameters, further characterization of OA-AC tissues may give additional information regarding the maintenance/restoration of ECM composition in the presence of N-TEC_CM. The study of further OA features, other than the here investigated inflammatory and catabolic components, could also complement the assessment of beneficial effects for example, if amelioration of senescent traits or impaired autophagy are found *(257–259)*.

For an in depth understanding of the potential disease modifying properties of N-TEC released factors, further studies would be required to investigate which components of the N-TEC secretome, including soluble molecules and extracellular vesicles, might be the triggers of the observed responses. Moreover, whether the pre-treatment of NC with specific molecules could boost such effects, as demonstrated for MSC in the context of a different pathology *(260)*.

Nonetheless, one of the main constraints for estimating the implications of the here described findings in a more physiological scenario, lies on the incomplete knowledge of underlying mechanisms, limiting the understanding of features that a relevant/standard model should involve. Among the ones here used, the pro-inflammatory component could be recapitulated mostly by

osteoarthritic synovial cells and the osteochondral explants. Those OA specimens were confirmed as the major contributors to the circulating levels of inflammatory cytokines, whereas most of the factors were not detected in the chondrocytes, either healthy (nasal or articular) or diseased (Tables 5, 6). Besides, secreted levels of the typically OA-associated factors IL1β, IL6, IL8, TNFα and RANTES were similar for all chondrocytes, and values of lower magnitude than in the OA-Syn and OA-OC (Tables 7, 8).

Collected data also reflects a wide variability between donors for all sources of OA specimens. This, in coherence with findings from other studies, with even higher number of experimental samples. In those, such variability is correlated with diverse the severity of pathological symptoms *(171, 174, 252)*. Higher cohorts of donors as well as clustering by age and/or grading of the disease, will presumably support sharper conclusions about the consistency of determined inflammatory and catabolic profiles in the different targeted populations.

Regarding the OA-phenotypes of the different cells/tissues here used, the transcriptomic/secretory profiles of OA-Syn and OA-OC, were confirmed consistent with previous reports *(193, 252)*. However, measured levels of cytokines such as IL1β, TNFα, IL8 and IL6 in OA-AC, were considerably lower than what described before on a different setup. In that same work, authors confirmed the significant effect that the culture of cells exerts on their secretory profile *(174)*. Therefore, taking into account that the OA-AC here utilized were expanded and chondrogenically differentiated, it is likely to deduce that such processing further reduced their pro-inflammatory profile, and that results might then differ from in vivo/in human conditions.

Overall, although the described results in this section indicate an anti-inflammatory and anti-catabolic potential of N-TEC secreted factors, the implications of such effects in an in human situation cannot be established by means of these in vitro approaches. Complementary investigations are required to better understand this potential.

Better setups would consider, for example, more physiological OA-environments such as one allowing the cross talk between joint tissues and cell types, using animal models or experimenting with more biomimetic models in vitro, such as bioreactors. However, all these alternatives will correspondingly add another level of complexity *(261)*.

4.3 Aim III: Compatibility of N-TEC within in vivo osteoarthritic environments. Survival, cartilaginous matrix maintenance and integration with surrounding tissues.

4.3.1 Introduction

A typical OA joint environment is characterized by metabolic cues such as the production of inflammatory and catabolic factors by resident cells in cartilage *(248, 262)*, synovial membrane *(263, 264)* and subchondral bone *(218, 265)*; in addition to the mechanical stimuli playing a role in the development and progression of the disease. In these OA-conditions no cell-based treatment have been yet considered physiologically suitable *(105, 221, 266)*, and for a successful therapy, both the survival of the graft - when exposed to the mentioned degrading conditions - as well as its integration with the surrounding cartilage and bone have to be demonstrated. This, as a critical condition to ensure stability of the joint, enable post-operative normal load distribution, and prevent future tissue degeneration *(267)*.

Evaluating survival and engraftment of any implantable structure, such as the nasal chondrocyte-based engineered tissues (N-TEC) studied in this dissertation, is not feasible by means of in vitro models (as the described for aims I and II). These culture systems have the constraint of do not recapitulate thoroughly the features of an osteoarthritic joint *(225)*, since they commonly involve only one of the afore mentioned components or an oversimplified version of them when combined.

Such modified representations may disregard, for example, the three-dimensional configuration or the tissue and/or the role of mechanical stimulation, neglecting the possible study of OA as multifactorial disease *(224)*.

With the aim of investigating the capacity of N-TEC to survive, integrate and maintain the cartilaginous matrix in a more physiological OA-environment, three different in vivo models were assessed in this thesis; two ectopic mouse models based on human osteoarthritic (i) engineered or (ii) explanted osteochondral tissues, and an (iii) orthotopic sheep model that was 'chronified' to mimic a degenerative scenario. This chapter summarizes the findings derived from assessing the performance of N-TEC in such OA-joint compartments simulated in vivo.

4.3.2 Results

4.3.2.1 Ectopic model of engineered OA-osteochondral tissues

4.3.2.1.1 N-TEC integrate and mature in vivo when combined with engineered bone compartments generated with osteoarthritic osteoblasts

The first in vivo approach assessed the maturation and integration of N-TEC within an OA-engineered osteochondral environment. N-TEC and bone-like tissues were separately engineered with GFP-labelled NC and OA osteoblasts (OA-OB), by pre-culture of the respective cells in chondrogenic or osteogenic medium during 3 to 5 days (Fig. 13A). Tissues were then combined to generate osteochondral constructs and ectopically implanted in mice for 8 weeks or maintained in vitro. After that time, N-TECs - both in vitro and in vivo - integrated with the underlying bone compartment (b) and produced cartilaginous matrix, as visualised by GAG staining with Safranin-O (Fig. 13B). Only in vivo constructs evidenced the novo deposition of bone matrix in the subchondral compartment, with typical features of embedded osteocytes and lining osteoblasts (arrow and arrowhead on Fig. 13B magnification).

Higher intensity of the staining in the in vivo setup, indicated further maturation of the tissues as compared to the in vitro condition (Fig. 13E). Implanted constructs also showed positivity for type II collagen localized on the expected cartilaginous layer, confirming the cartilaginous composition of the deposited ECM (Fig 13C). In the same condition, type I collagen was only identified in the non-cartilaginous subchondral regions and in the superficial zone of the cartilage compartment, where remnants of the matrix material (composed of type I/III collagen) are expected (Fig. 13D). ECM of in vitro maintained constructs was not homogenously deposited and negative for the chondrogenic ECM marker collagen type II (Fig. 13F, G).

The results obtained with this model indicate that N-TEC further mature in this *in vivo* environment when not fully differentiated prior implantation, maintain the deposited cartilaginous ECM, and integrate to OA-osteoblast-based (or containing) bone compartments.

4.3.2.1.2 N-TEC remains avascular and contribute to the formation of integrative tissues when combined with engineered bone compartments generated with osteoarthritic osteoblasts in vivo

After verifying the integration and maturation of N-TEC within the engineered OA-osteochondral model, its capacity to remain avascular (as one of the features of healthy cartilage) and the role of the implanted cells in the integrative process were further investigated. To identify blood vessels, laminin staining was performed, and such structures found only in the bone compartment while not in the N-TEC zone (Fig. 14A). This demonstrating the capacity of N-TEC to mature into avascular cartilaginous tissues, even in close proximity with OA-osteochondral engineered tissues in vivo. To track cell fate 8 weeks after implantation, their human origin was identified by *Alu in situ* hybridisation throughout the entire construct (Fig. 14B). GFP-labelled cells were localized in the cartilaginous region and in the intermediate region between cartilage and bone (Fig. 14C), which

indicates that implanted NC participate in the formation of integrative tissue between the N-TEC and the bone compartment.

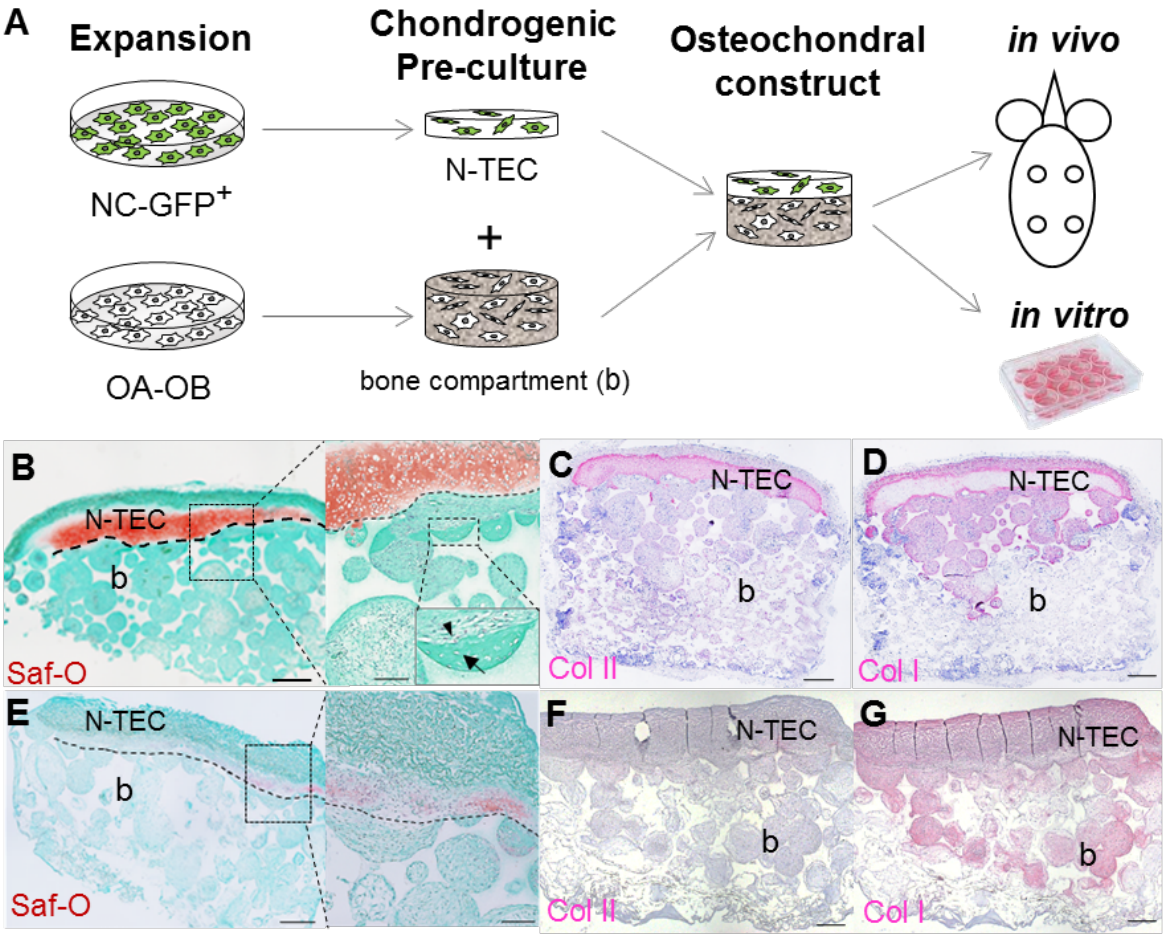

Figure 13. Engineered OA-osteochondral constructs and their matrix deposition in vitro and in vivo. (**A**) Schematic representation of in vitro construct generation, by a combination of N-TEC (containing GFP^{+}-NC) with a bone compartment (containing OA-OB) for posterior implantation in mice or in vitro culture. Safranin-O staining of (**B**) constructs after 8 weeks of implantation or (**E**) in vitro maintenance. Magnification of **B** shows the presence of an osteocyst (arrow) and a lining osteoblast (arrowhead) of an osteoid in the bone compartment. Collagen type II (**C,F**) or type I (**D,G**) immunostaining for constructs after 8 weeks of implantation, or in vitro maintenance respectively. N-TEC= Nasal chondrocyte-based tissue engineered cartilage, b= bone compartment. Scale bar: 500 µm; 100 µm (magnifications on **B,E**).

4.3.2.2 Ectopic model of OA-osteochondral explants

4.3.2.2.1 N-TEC integrate and maintain cartilaginous matrix in vivo, when combined with OA-osteochondral explants

Following the in vivo assessment into a simplified OA-osteochondral model (involving a single cell type, i.e., OA-OB), N-TEC performance was evaluated within osteoarthritic-osteochondral tissue explants (OA-OC), containing native OA cartilage and bone, as well as the corresponding cell types. Cartilaginous tissues were engineered with GFP-labelled NC, similarly to clinical manufacturing of human N-TEC *(157)*. Following its generation, they were placed into cartilage defects, created in osteochondral tissues explanted from end-stage OA joints. Resulting constructs were either maintained in vitro, or subcutaneously implanted in mice for 8 weeks (Fig. 15A). Both in vitro (Fig. 15B) and in vivo (Fig. 15C) constructs deposited cartilaginous matrix, as observed by GAG detection with Saf-O staining.

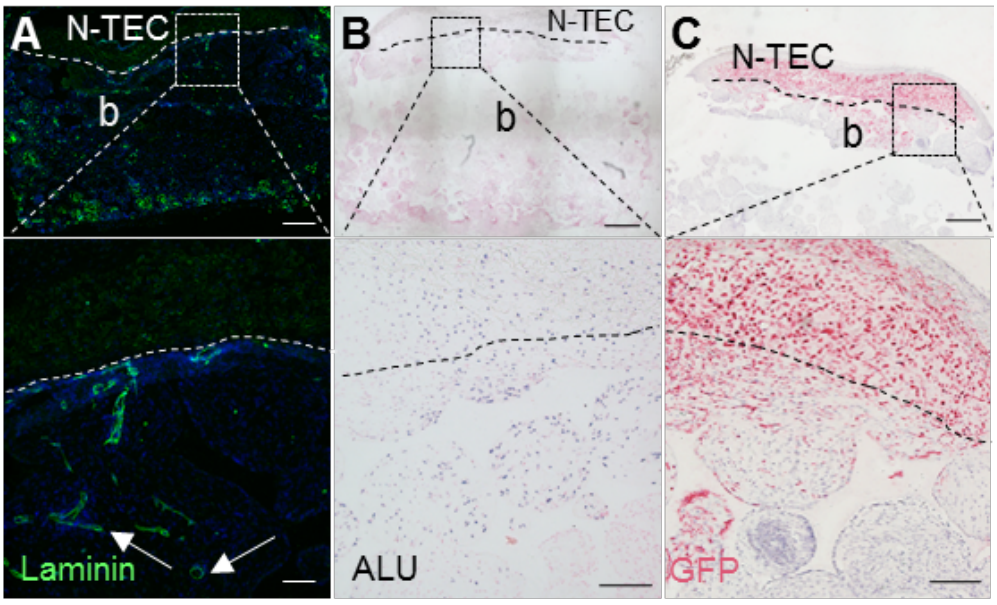

Figure 14. Characterization of implanted engineered OA-osteochondral constructs. (**A**) Identification of vessels (pointed with arrows) in the osteogenic compartment by laminin immunofluorescence. (**B**) in situ hybridization for ALU sequence labelling human cells. (**C**) Immunohistochemical detection of GFP-positive NC. Pictures in bottom row show the magnification of indicated areas. N-TEC= Nasal chondrocyte-based tissue engineered cartilage, b= bone compartment. Scale bar: 500 µm (upper pictures); 100 µm (bottom pictures).

However, whereas in vitro N-TEC grafts remained disconnected from the surrounding osteochondral tissues, they integrated in vivo with the adjacent native cartilage and subchondral bone after 8 weeks of ectopic implantation (Fig. 15 D,E). Arbitrary scoring of integration (from absent=0 to complete=3) evidenced that, while in vitro maintained tissues displayed only poor integration with the surrounding cartilage, implanted constructs integrated to both cartilage and underlying bone, with a higher scored achieved between N-TEC and cartilage (Table 9). Inferior, but evident, integration between the graft and bone could be attributed to technical artefacts, such as the rudimentary preparation of the defects, leading to non-smooth interfaces (Fig. 1F). Further characterization of implanted OA-OC tissues evidenced the presence of MMP13 positive regions, indicating ongoing OA-related catabolic processes (Fig. 15G). In the graft area, cartilaginous composition of the deposited ECM was demonstrated by type II collagen positivity, while type I collagen was additionally detected as one of the main components of the scaffold (Fig. 15H).

Table 9. Integration score for N-TEC within OA-osteochondral explants in vivo. Scoring of the integration between the graft (N-TEC) with the surrounding cartilage and underneath bone for in vitro maintained or in vivo implanted constructs. Score represents and arbitrary scale for no integration=0, poor integration=1, partial integration=2 and complete integration=3. Values presented as mean ±SD of n=5 in vitro constructs and n=6 in vivo samples.

	N-TEC - cartilage	**N-TEC - bone**
in vitro	0.8 ± 0.4	0.0 ± 0.0
in vivo	2.7 ± 0.6	1.8 ± 1.0

To investigate the role of implanted cells in the formation of the integrative tissue, presence of human cells in the interfaces between N-TEC and both native cartilage and subchondral bone, was confirmed by Alu *in situ* hybridization (Fig. 16 A,B). Detection of GFP^+ cells by

immunofluorescence evidenced the localization of implanted NC in the interstitial zone (Fig. 16C). All these data confirming, in a more physiological model than the previous engineered OA-osteochondral tissue model, the capacity of N-TEC to integrate and maintain a cartilaginous matrix, as well as the ability of NC to survive and contribute to the integration process when placed into OA-environments *in vivo*.

4.3.2.3 Orthotopic Sheep model of induced OA

4.3.2.3.1 Non treated articular lesions, induced in sheep joints, chronify and show OA signs

After assessing the compatibility of NC within ectopic in vivo environments, integration of N-TEC and survival of the implanted cells were investigated in a load-bearing OA joint environment, recreated in an orthotopic sheep model. OA was induced in sheep joints (condyles) by generating full depth cartilage defects and leaving them untreated for 8 weeks (Fig. 17A). After OA induction, cartilage tissue in regions where the defects were created, presented macroscopic osteoarthritic marks, such as uneven and fibrous-like surface (Fig. 17B). Histological assessment confirmed lack of cartilage-specific proteoglycans in the tissue biopsies, received from the debrided areas (Fig. 18C). Successful induction of OA was additionally demonstrated by the amount of synovial fluid which could be collected in higher amounts for the defected joint, as compared to the healthy one (0.52 ± 0.45mL vs 0.17 ± 0.21mL, respectively). In the collected samples, a trend of increase in levels of the pro-inflammatory/catabolic factors IL1β, TNFα, MMP1 and IL8 was observed (Fig. 17D). No changes in the levels of additionally measured factors were detected, when comparing healthy and OA joints (Table 10).

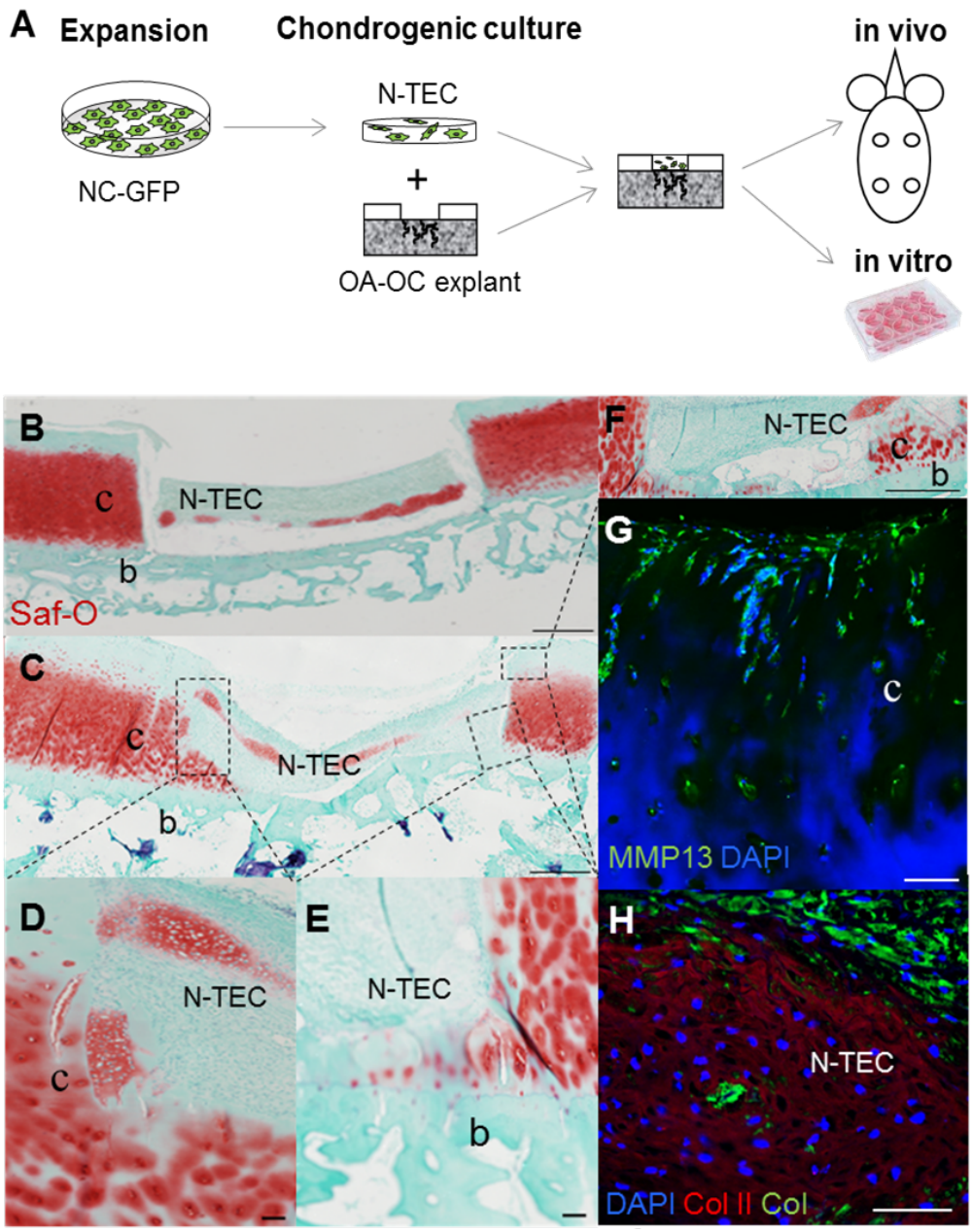

Figure 15. Ectopic implantation of N-TEC grafts combined with osteochondral explants. (**A**) Schematic representation of human osteochondral construct formation, by combination of N-TEC (generated with GFP-labelled NC) within a cartilage defect in an osteoarthritic osteochondral explant (OA-OC). Formed constructs were maintained in vitro or subcutaneously implanted for 8 weeks in vivo. (**B**) Safranin-O staining of the construct after 8 weeks at control conditions in vitro or (**C**) in vivo. Magnification of indicated areas for Safranin-O staining of (**D**) N-TEC-cartilage or (**E**) N-TEC-bone. (**F**) Safranin-O staining for a region with poor integration between N-TEC and the underlying bone compartment. (**G**) MMP13 immunofluorescence of implanted osteochondral tissues. (**H**) Immunofluorescent co-localisation of type II (red) and type I (green) collagen. N-TEC= Nasal chondrocyte-based tissue engineered cartilage, c= native cartilage, b= subchondral bone. Scale bar: 1mm (B,C), 100µm (D-H).

These observations indicate that the induction of OA-like defects, can be achieved after creation of defects on sheep joints, if left untreated. Such OA features, evidenced by macroscopic, histological and secretory changes, could provide and osteoarthritic environment in which to assess the performance of potential therapeutic products such as N-TEC.

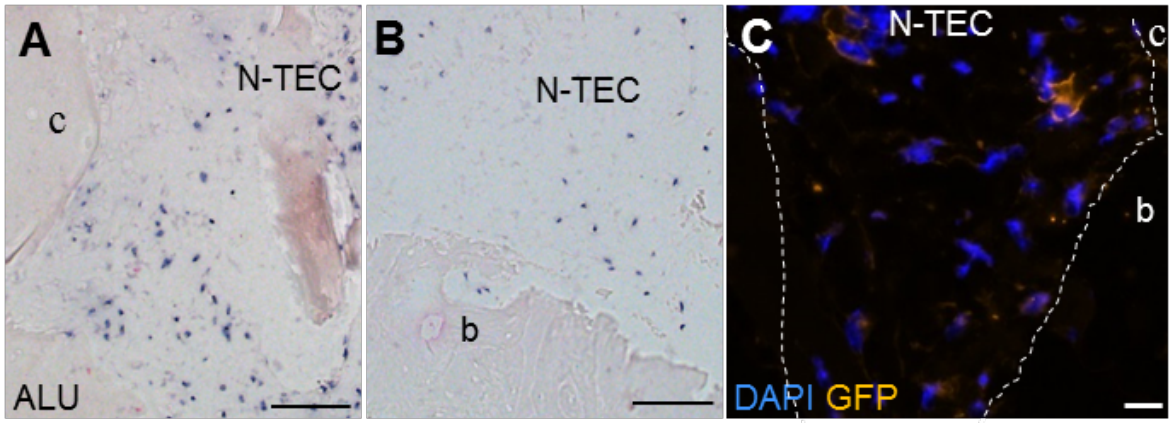

Figure 16. Presence of implanted nasal chondrocytes in the interstitial tissue. Human origin of cells confirmed by dark nuclear staining in (**A**) N-TEC and native cartilage, or (**B**) N-TEC and bone interfaces. (**C**) Detection of implanted cells by GFP immunofluorescence. Scale bar: 100µm.

4.3.2.3.2 Engineered cartilaginous tissues- based on sheep nasal chondrocytes (sN-TEC) can be generated and their properties preserved upon implantation in OA-induced defects

Parallel to defect creation, sheep NC (sNC) were isolated from the same animal, GFP-labelled (transduction efficiency of 96.0-99.9%), expanded and used for the engineering of cartilage grafts (sN-TEC), similarly to clinical manufacturing of human N-TEC *(157)*. After generation, grafts were implanted in the OA-induced defect sites and their performance assessed after 4 and 12 months (Fig. 17A). Generated sN-TEC tissues, whose cartilaginous composition was confirmed by positive staining for GAG (Fig. 18A) and type II collagen (Fig. 18B), were implanted into the created OA-like defect. At the time of explantation, grafts were well-integrated into the recipient site and no further degeneration or signs of inflammation were macroscopically seen in the N-TEC

treated joints (Fig. 18 C,E). In coherence with the typical variability of large animal models, the repair tissue displayed various quality patterns (best and worst specimens shown in Fig. 18E).

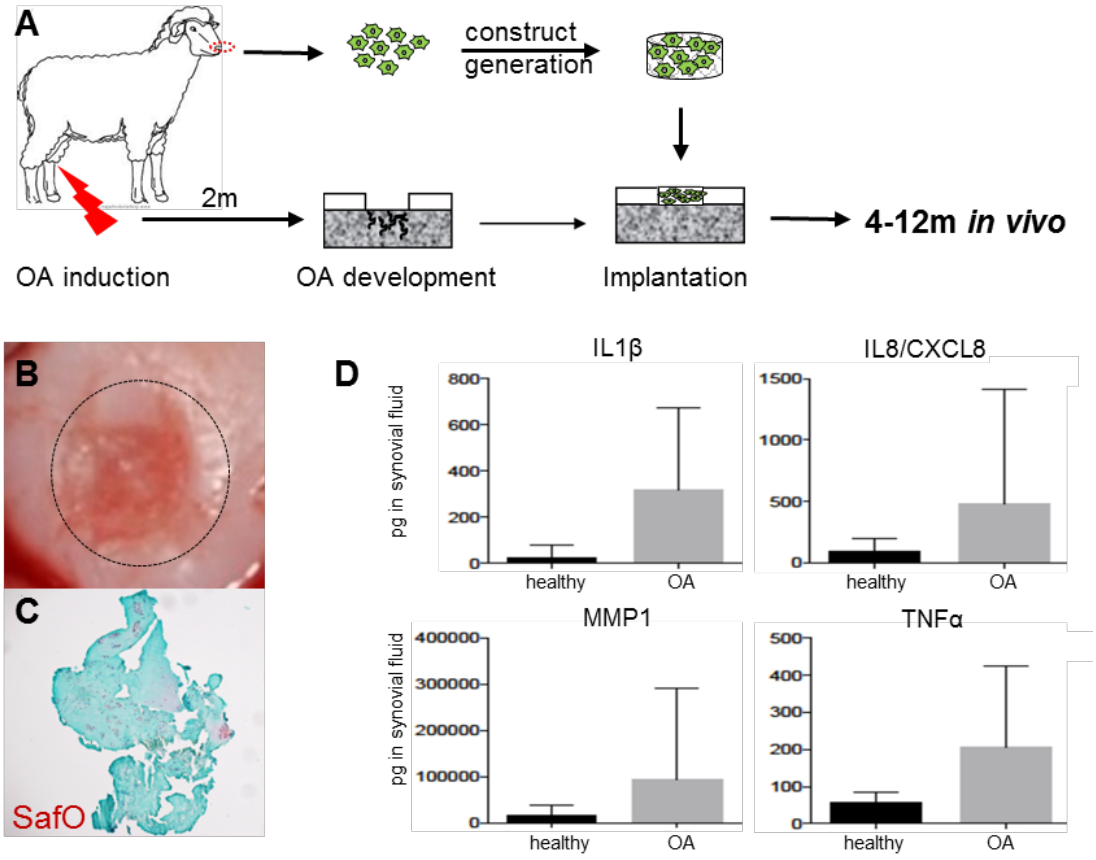

Figure 17. Experimental design and induction of OA-cartilage defects in sheep. (A) Experimental design of the large animal study. Sheep nasal chondrocytes (sNC) were GFP-labelled and seeded on ChondroGide® scaffolds to manufacture cartilaginous grafts (sN-TEC) that were further implanted into OA-induced cartilage defects for up to 12 months. (B) Appearance of the osteoarthritic joint after OA induction. Circles indicate the area of debridement prior to graft transplantation. (C) Safranin-O staining of the debrided OA cartilage. (D) Protein levels of factors present in the synovial fluid of healthy and OA sheep (n=6 animals at each time point). Measured concentrations (pg/mL) were normalized to the volume of synovial fluid, per sample. Scale bar: 100µm.

Table 10. Inflammatory and catabolic factors on sheep synovial fluid. Concentrations for factors quantified in the synovial fluid of sheep (n=6) prior OA induction (=healthy), 8 weeks later at the time of N-TEC implantation (=OA) and 4-12 months after treatment (=treated). Values (in pg) represented as mean ±SD. Concentrations (pg/mL) were measured and normalized to the volume per sample. * indicates a significant difference of the treated group to the OA group defined by Mann-Whitney U test. Unilateral $p<0.05$ considered significant.

	healthy	OA	treated
IL1β	24.1 ± 53.9	317.5 ± 355.5	169.3 ± 338.6
IL4	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
IL6	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
IL7	14.9 ± 29.7	1.1 ± 2.7	0.0 ± 0.0
IL8/CXCL8	98.5 ± 104.7	488.0 ± 922.5	38.8 ± 44.9*
IL10	7.9 ± 6.0	13.9 ± 13.3	6.0 ± 3.4
IL13	0.0 ± 0.0	35.8 ± 55.6	16.6 ± 33.3
TNFα	58.3 ± 27.9	206.9 ± 218.3	44.3 ± 49.2*
MMP1	15229.6 ± 23556.8	93770.5 ± 196595.8	13137.7 ± 24476.7
MMP3	364.0 ± 813.8	5813.2 ± 9542.2	537.7 ± 860.4
MMP13	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
ADAMTS5	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0

Volume of the synovial fluid obtained following the treatment was significantly decreased (0.17±0.16mL vs 0.52±0.45mL ($p=0.047$)), reaching the range of healthy joints (0,17 ±0.21mL). In one of the animals assessed at 12 months, no synovial fluid could be harvested at all. Some inflammatory cytokines in the collected synovial fluid samples decreased to ranges detected in healthy sheep (i.e., MMP1, MMP3, IL1β, IL10/CXCL10, IL13) (Table 10), or were significantly decreased as compared to the stage after OA induction and before treatment (i.e., IL8/CXCL8 and TNFα) (12.57-fold and 4.67-fold change respectively) (Fig. 18D).

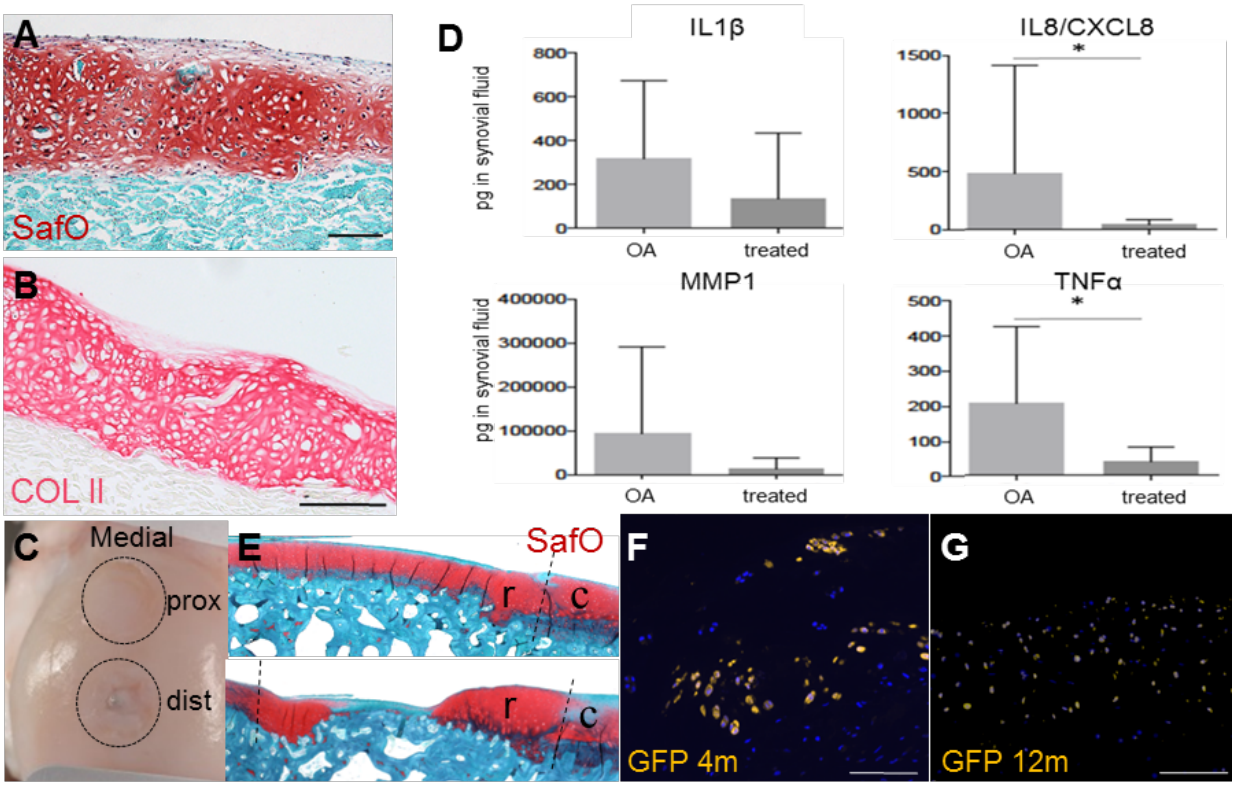

Figure 18. Orthotopic implantation of sN-TEC into osteoarthritic cartilage defects in sheep. Quality of a representative sN-TEC prior implantation, (**A**) stained with Safranin-O and (**B**) assessed for type II collagen deposition. (**C**) Macroscopic appearance of cartilage repair at 12 months post implantation. (**D**) Protein levels of factors present in the synovial fluid of OA and N-TEC treated sheep (n=6 animals at each time point). Measured concentrations (pg/mL) were normalized to the volume of synovial fluid, per sample. (**E**) Histological staining (Saf-O) for best (upper) and worst (bottom picture) cartilage repair within the osteochondral tissues. Identification of implanted GFP positive sNC in explants after (**I**) 4 and (**J**) 12 months. Scale bar: 100µm. * = significantly different ($p<0.05$) between indicated groups, as assessed by Mann-Whitney-U tests.

Implanted sNC could be tracked and identified by GFP immunofluorescence after 4 and 12 months (Fig. 18F, G). Cells were present both in the region of repair cartilage (r) (where grafts were presumably implanted, since borders were not always evident), and in the adjacent native cartilage (c) (Table 11). These results indicate sustained survival of implanted sNC in an OA joint and the capacity of sN-TEC to maintain cartilaginous features, while integrating with the surrounding tissues.

Findings described in this chapter demonstrate that when N-TEC is implanted in both ectopic and orthotopic in vivo OA-models, nasal chondrocytes can survive and contribute to the integration with adjacent tissues, while preserving the cartilaginous matrix. Moreover, and consistent with the findings of *section 4.2* (*Aim II*), on treated joints of the orthotopic model, levels of circulating inflammatory factors were dampened, presumably due to the presence of N-TEC - or its secreted factors –.

The in vivo models hence used, confirm the capacity of N-TEC to withstand OA-inflammatory conditions (*Aim I*) and to modulate the secretory profile of OA-cells (*Aim II*). In addition, the results summarized in this chapter demonstrate the compatibility of N-TEC within in vivo setups, in terms of integration with surrounding OA-tissues and further maturation of the implanted engineered cartilage. All these data suggest that in the context of an osteoarthritic joint, N-TEC could add to structural stability and reduce circulating levels of inflammatory and catabolic factors, which could have a potential disease modifying effect in long term.

Table 11. Quantification of GFP positive (GFP+) cells in sheep condyles following N-TEC implantation. Ratio of the number of GFP+ cells (implanted nasal chondrocytes (NC) over total cells (stained in DAPI) for the indicated regions within the joints. Cells were quantified for two independent histological sections of each treated joint (n=8 constructs at 4 months and n=16 constructs at 12 months).

	4 months	**12 months**
% GFP+ cells / total cells in cartilaginous layer	31.6 ± 17.5	23.9 ± 10.6
% GFP+ cells in graft / total cells in graft	17.4 ± 15.3	10.6 ± 5.8
% GFP+ cells in native cartilage / total cells in native cartilage	14.2 ± 10.9	13.3 ± 9.9
% GFP+ cells in graft / total GFP+ cells in cartilaginous layer	49.6 ± 25.9	48.2 ± 22.5

4.3.3 Discussion

Since no reliable pre-clinical models exist to predict the efficacy of cartilage repair in clinical OA settings, in this thesis different models were employed to address specific questions that could not be answered with the previously described in vitro models (*sections 4.1, 4.2*), and that must be investigated when clinical application of a potential therapeutic agent is envisioned.

In all in vivo models, it was demonstrated the capacity of nasal chondrocyte-based tissues to integrate with adjacent tissues and preserve the cartilaginous ECM, as required features for any structure to be implanted in an OA-environment. More specifically, the ectopic mouse model of engineered OA-osteochondral tissues, demonstrated that N-TEC could remain phenotypically stable, avascular, and efficiently integrate with underlying subchondral. The also ectopic, mouse model of human OA-osteochondral explants, could evidence cell survival and participation of the implanted cells in the formation of integrative tissue between N-TEC and both the OA-cartilage and subchondral bone. In this case, although the expression of a typical marker of cartilage degeneration in OA, MMP13 *(268, 269)*, was still maintained in the OA osteochondral samples upon implantation (Fig. 15G), additional OA features such as secretion of inflammatory factors might not be preserved in such ectopic environment and therefore must be further confirmed. Complementary analysis such as immunohistological detection of pro-inflammatory and catabolic molecules, as well as secretory analysis of explanted tissues, could contribute to a better clarification for the maintenance of the OA-like environment.

In these ectopic environments, absence of mechanical load and presumable loss of the OA-inflammatory status are not fully representative of the pathological features of an OA in vivo environment. Therefore, a large animal model of induced OA was therefore included with the aim of demonstrating the survival of implanted cells in an orthotopic site (load bearing), and for an

extended time than the assessed in ectopic models. Described results corroborate that implanted NC were detectable for at least 1 year after implantation in both the graft and the native cartilage areas, suggesting their participation into cartilage regeneration. In addition, this model allowed the analysis of synovial effusions, in which levels of inflammatory cytokines were reduced following the treatment with N-TEC. These findings support the indications of anti-inflammatory effect exerted by N-TEC released factors, as demonstrated when applied to OA-cells/tissues in vitro (Aim II).

The large animal model of OA used in this study, constituted the more physiological environment employed in the entire study. In fact, the successful creation of an OA-like defect in these animals, could be confirmed by the increased volumes of synovial effusions of treated joints, as typical reported in human OA *(171, 251, 270, 271)*. Increased secreted levels of pro-inflammatory and catabolic factors (IL1β, TNFα, MMP1 and IL8) also evidenced this OA-environment, as reported and observed in other studies.

The here used ovine/sheep, is among the more commonly used and recommended to investigate orthotopic cartilage repair *(234)*, and it have been employed in different setups, concerning the evaluation of MSC-based approaches for cartilage repair *(272)*. Still, it is crucial to highlight that findings derived from this and other large animal studies, are not predictive of the clinical outcomes in patients *(233, 273, 274)*, mostly due to not only differences in biomechanics and physiology between species, but also to species-specific dissimilarities in disease pathology and progression, normal joint homeostasis and repair capacity *(224, 274)*.

Controlled clinical investigations in patients must be conducted to ultimately understand the performance of the here used N-TEC, in terms of human safety and therapeutic efficacy for the targeted lesions. Previous investigations of the group have demonstrated already safety and

feasibility of N-TEC in the context of cartilaginous focal lesions *(157)*. Therefore, studies addressing efficacy and a standardised manufacturing will need to be conducted in order to bring this potential TE therapy into clinical practice *(275)*.

In regards to the capacity of NC to acquire the molecular identity of an implantation site *(section 3.3)*, it is important to consider that some alterations in the expression of factors such as the HOX genes are also reported in OA *(276)*, and in particular in OA-AC that would key components of the implantation environment within an OA lesion *(277)*. Assessing whether this altered pattern can be also acquired by NC, will be informative concerning a possible genetic limitation for the long-term performance of N-TEC under OA conditions, compromisingthe beneficial outcomes widely described in this whole thesis.

5 Conclusions

Aim I. *Determining the capacity of N-TEC to form and maintain cartilaginous properties, when exposed in vitro to factors secreted by OA cells/tissues and pro-inflammatory cytokines.*

- Nasal chondrocyte-based engineered cartilaginous tissues (N-TEC) can maintain their tissue properties (determined by the content of cartilage ECM molecules, GAG and type II collagen) under inflammatory conditions simulating the onset of osteoarthritis (OA). When compared to A-TEC, this OA-withstanding capacity is superior for N-TEC in terms of ECM composition.
- Effective triggering of a higher inflammatory profile – as in OA -, can be obtained in both nasal and articular derived tissues, by exposure to a chemically defined 'low inflammation' cocktail, as well as to medium conditioned by osteoarthritic synovial cells; as evidenced by the significant increased secretion of OA-associated markers IL6, IL8, TNFα and RANTES.

Aim II. *Evaluating the potency of N-TEC released factors to damper the production of inflammatory and catabolic molecules in OA cells/tissues in vitro.*

- Factors released by N-TEC can damper the inflammatory and catabolic profile of OA cells/tissue in a target specific manner:
  (i) In osteoarthritic articular chondrocytes (OA-AC) by significantly reducing gene expression levels of *IL8*, *MMP13* and *ADAMTS5,* as well as MMP13 content.
  (ii) In osteoarthritic synovial cells (OA-Syn) by reducing gene expression levels of *IL8*, *MMP13* and *ADAMTS5* (similarly to the effect in OA-AC), and by down regulating the secreted concentrations of OA-associated markers (IL6, IL8, TNFα and RANTES).
  (iii) In osteoarthritic osteochondral explants (OA-OC) by down regulating the secreted levels of OA-associated markers (TNFα, MMP3 and ADAMTS4).

– The N-TEC secretome exert superior anti-inflammatory/catabolic properties than A-TEC, when applied to OA-AC and OA-Syn. In opposition, A-TEC released factors can exacerbate the OA environment, by increasing the secretion of IL6 and IL8 in OA-AC (while N-TEC does not) and not down regulating the concentrations of IL6 and IL8 in OA-Syn (whereas N-TEC does).

Aim III. *Assessing the suitability of N-TEC for OA-environments simulated in vivo, in terms of cell survival, cartilaginous matrix maintenance and integration with surrounding tissues.*

- N-TEC can survive and integrate to surrounding tissues in different in vivo OA simulated models. Additionally, nasal chondrocyte-based tissues are able to:
  (i) Further mature and remain avascular (as evidenced in the ectopic model of engineered osteochondral tissues)
  (ii) Participate in the formation of integrative tissue within the graft (N-TEC) and both OA-cartilage and underlying bone (as demonstrated in the ectopic osteochondral explant and sheep model)
  (iii) Maintain the previously described features for up to 12 months in a loaded orthotopic model of induced OA (in sheep), while reducing the circulating levels of the pro-inflammatory cytokines IL8 and TNFα, consistently to the observations in the in vitro models.

6 Future perspectives

The findings derived from my PhD thesis indicate that nasal chondrocyte-based engineered tissues (N-TEC) can be indeed envisioned as suitable treatment of osteoarthritic defects, regarding their well confirmed capacity to preserve the cartilaginous properties under diverse OA simulated conditions, confirmed so far in vivo for up to 12 months (in the sheep model). In fact, due to the positive results here described, and considering the previously demonstrated safety and feasibility of N-TEC to treat post-traumatic cartilage defects (published by our group in *The Lancet*, 2016); two young patients diagnosed with medial compartmental OA, have been treated with autologous N-TEC, having positive outcomes for up to 22 months (which is the latest available follow up).

These two clinical cases were intervened following authorization by Swissmedic, and otherwise considered for prosthetic joint replacement (not indicated for their age range). The main outcomes included no adverse reactions and formation of repair tissue at the level of the surrounding cartilage, as well as reduction in self-reported scores of pain and disability. These results, along with the in vitro/in vivo data comprised in this thesis, have been submitted to the journal of *Science Translational Medicine,* and is currently under revision.

Regarding this clinical application of N-TEC in OA, it is important to bear in mind that there are not animal models accepted by competent authorities as predictive of the clinical efficacy for this disease. Therefore, further investigations should focus on the efficacy assessment in the frame of a phase II study, in terms of the evaluation of the long-term performance of the graft, and in the clarification of the neat effect of N-TEC in the mentioned outcomes, since additional mechanical correction was performed along in both patients. However, although additional surgical manipulation can influence the recovery of structural aspects in the joint, it is not expected to have any disease modifying capacity. This consideration is relevant in view of the anti-inflammatory and anti-catabolic features displayed by N-TEC, both in vitro and in vivo. Such potential could

provide, for the first time, a possible strategy for disease-modification of OA, thus avoiding or delaying the irreversible intervention of joint replacement. Further investigations including the assessment of presence and composition of synovial fluid, along with the evaluation of the fibrotic or hyaline character of the formed repair tissue, could be the envisioned strategy to elucidate the long- erm scope of these results.

In view of further trials, the potential efficacy of N-TEC for the repair of OA-defects must be also investigated in comparison to commonly used treatments such as the injections of hyaluronic acid. This would be helpful in order to establish whether the implantation of N-TEC can improve the clinical outcome over those standard treatments. In addition, the possibility of combining N-TEC with other treatments/drugs could be contemplated as a strategy to counteract the appearance of adverse events such as hypertrophy.

Considering the variety of disease etiologies, either from single or multiple factors, extremely heterogeneous patient groups are found, and it is rather unlikely that one treatment can be effective for all patients. Therefore, it is also important to identify biomarkers that can be used for selection of ‘responders’ to a certain therapy, such as N-TEC implantation. Prediction of outcomes before treatment would avoid unnecessary risks for the patient, and lead to reduction of health care costs, as treatment would only be attempted in potentially responsive patients. In addition, identification of biomarkers and understanding of the associated pathways will aid in the development of improved treatment options and in the understanding of the disease mechanisms.

Foreseeing a better understanding of the biology of nasal chondrocytes (NC) and their distinct nature from articular (AC) ones, a comparative transcriptomic profiling been performed by our group, identifying 1,726 genes differentially expressed between both cell types. In the context of the inflammatory focus of my research, 17 of these differentially expressed genes have been

detected as related to inflammation, suggesting a differential role in the OA environment. However, the establishment of differential features related to other pathways important for cartilage formation such as Wnt (wingless/integrated-1) and BMP (bone morphogenic proteins), among others, will require further analysis and investigation.

7 Materials and Methods

7.2 Materials

7.2.1 Table 12. Equipment

Item	Reference	Manufacturer
Elisa reader	Synergy H1 Hybrid Multi-Mode Reader	BioTek
Tissue processing center	TPC 15 Duo	Medite
Tissue embedding station	TES Valida	MEDITE
Sliding Microtome	HM 430	Thermo Fisher Scientific
Inverted microscope	Eclipse Ti2	Nikon
Luminex Reader	Bio-Plex 200	Bio-rad
Real-Time PCR cycler/detector	Applied Biosystems 750	Thermo Fisher Scientific
Flow cytometer	Accuri C6	BD biosciences

7.2.2 Table 13. Cell supports/Scaffolds

Material	Reference	Manufacturer
Non-adherent U bottom 96-well plates	268200	Thermo Fisher Scientific
Collagen-based sponge	ChondroGide,	Geistlich Pharma AG
Ceramic scaffold	Engipore	Finceramica

7.2.3 Media composition

Table 14. *Basic medium*

	Reference	Manufacturer
Dulbecco's modified medium (DMEM)	10938-025	Invitrogen
10 mM HEPES	15630-056	Invitrogen
1 mM sodium pyruvate	11360-039	Invitrogen
100 U/ml penicillin-streptomycin-glutamine (PSG)	10378-016	Invitrogen

Table 15. *Chondrogenic medium*

Based on *basic medium* and with the following supplements

			For differentiation in	
	Reference	Manufacturer	micro/macro-masses	Collagen based-scaffold
20 % Human serum albumin (HSA)	43075	CSL Behring	x	
10ng/mL TGF-β3	-	Novartis	x	
50mM Ascorbic acid (AA) -	A-8690	Sigma	x	x
Insulin-Transferrin-Selenite (ITS)	51300-044	Gibco	x	
Linoleic acid	L9530-5	Sigma	x	
10^{-7}M Dexamethasone	D-2915	Sigma	x	
10µg/mL Insulin	Actrapid HM	Novo Nordisk Pharma AG		x
5% FBS*	10270-106	Invitrogen		x

* For manufacturing of the N-TEC implanted in patients, autologous serum used instead.

Table 16. *Osteogenic medium*

	Reference	Manufacturer
Minimum Essential Medium **α (**αMEM)	22571-020	Invitrogen
10% FBS	10270-106	Invitrogen
0.1 mM Ascorbic acid (AA)	A-8690	Sigma
10 mM Sodium beta glycerolphosphate pentahydrate	G-9891	Sigma
10 nM Dexamethasone	D-2915	Sigma

7.2.4 Table 17. Antibodies

		Dilution	Reference	Manufacturer
Primary	Type II collagen	1:5000	34712	Abcam
	Type I collagen	1:1000	138492	Abcam
	GFP	1:1000	1020	Aves Lab
	MMP13	1:500	39012	Abcam
	Laminin	1:250	11575	Abcam
Secondary	Alexa Fluor 568	1:200	A11041	Thermofisher Scientific
	Alexa Fluor 488	1:200	A11008	Thermofisher Scientific
	Alexa Fluor 647	1:200	A21241	Thermofisher Scientific
	Biotinilated AB for GFP IHC	1:300	BA-1400	Vector Laboratories

7.2.5 Table 18. Primers and probes

	Reference TaqMan™ assay	Manufacturer
COL2	Hs00264051	
COL1	Hs00164004	
ACAN	Hs00153936	
MMP13	Hs00233992	
MMP3	Hs00968305	Thermofisher Scientific
ADAMTS5	Hs00199841	
IL8	Hs00174103	
IL6	Hs00985639	
GAPDH	Hs02758991	

7.2.6 Table 19. Detection assays

	Reagent	Reference	Manufacturer
Biochemical methods	Dimethylmethyleneblue (DMMB)	341088	Sigma
	Chondroitin sulfate	C9819	Sigma
	Bacteriophage λ DNA CyQuant	C7026	Invitrogen
Staining	Safranin-O	84120	Sigma
	Fast-Green	F-72552	Sigma
	Hemaetoxylin	3873	J.T. Baker
	Goat serum	16210-064	Invitrogen
	Vectastain kit (phospathase-substrate)	AK-5000	Linaris
	Hyaluronidase	H3884	Sigma
	Dapi	DI306	Thermofisher Scientific
	Pronase	10 165 921001	Roche
Gene expression analysis	Quick-RNA™ Miniprep Plus Kit	R1054	Zymo Research
	SuperScript™ III reverse transcriptase	18080044	Invitrogen
Protein assays	Luminex kit	Magnetic customized Assay	R&D Systems
	Human MMP13 ELISA	Sensolyte® Plus 520 Assay	Anaspec
	Human ADAMTS4 and 5 ELISA	Duo Set DY219805, DY430705	R&D Systems
	Sheep ELISA IL1β, IL6, IL8/CXCL8, IL10, TNFα, IL4, IL7, IL13, MMP13	(MBS734810),(MBS760504),(MBS025204),(MBS704435),(MBS763586),(MBS736299),(MBS925047),(MBS043750), (MBS2511038)	My BioSource
	Bovine ELISA MMP1, MMP3, ADAMTS5	(MBS031074),(MBS9310794), (MBS933409)	My BioSource

7.2.7 Table 20. Additional reagents

Reagent	Reference	Manufacturer
Type II collagenase	4176	Sorthington
Trypsin	25300-096	Invitrogen
IL1β	I9401	Sigma
TNFα	H8916	Sigma
IL6	206-IL	R&D Systems
Protein kinase	P2308	Sigma Aldrich
PBS	20012-068	Invitrogen
EDTA	S4881	Sigma
Fibrin glue	Tisseel	Baxter

7.3 Methods

7.3.1 Cell biology methods

7.3.1.1 Regulatory compliance for human and animal studies

All human samples were collected with informed consent given by the involved individuals and/or relatives. All animals were treated in agreement with the Swiss legislation with approval by the Veterinary Office of Canton Basel-Stadt (permission # 1797, for experiments with mice) and the responsible veterinary office of Zürich (Kantonales Veterinäramt Zürich, for sheep studies). Studies on human patients were approved by EKNZ (the cantonal ethical authority of Basel Ethikkomission Nordwest- und Zentralschweiz) and by Swissmedic (Swiss Regulatory agency for therapeutic products).

7.3.1.2 Cell and tissue isolation

7.3.1.2.1 Chondrocytes

Nasal chondrocytes (**NC**) were isolated from the nasal septums (n=14) of human donors obtained as residual tissue from surgical interventions or post-mortem. Healthy articular chondrocytes (**AC**)

(n= 9), were taken from regions of healthy-looking cartilage (i.e., no fibrous, soft or vessel invaded areas) from joints of patients undergoing engineered cartilage implantation, or from articular tissues harvested post-mortem. For osteoarthritic articular chondrocytes (**OA-AC**), cells were isolated from evidently degenerated joints from patients undergoing knee replacement (n=9).

Cartilage from all sources was mechanically dissected and enzymatically digested with collagenase (1.5mg/mL, 22 hours). After, enzyme was washed away by centrifugation (1500rpm, 3min) and cells plated and expanded for up to 3 passages in expansion medium (basic medium containing 1ng/mL TGF-β1 and 5ng/mL FGF-2), using culture flasks and at a seeding density of 5.000 to 10.000 cells/cm^2. All cultures were maintained at 37°C and 5% CO_2 in this and further steps.

7.3.1.2.2 Osteoarthritic cells and tissues

All OA tissue biopsies were collected from patients undergoing total knee replacement. ***OA-Syn*** (n=12) and ***OA-OB*** (n=1) were obtained by outgrowth culture of synovial membrane and subchondral bone biopsies, respectively, and cultured as previously described *(252, 278)*. ***OA-OC*** *(n=3)* were isolated from tibias, chopped into pieces and either kept in control medium (basic medium 2% FBS) (CTR) overnight - prior exposure to the corresponding treatments -, or maintained in osteogenic medium for production of conditioned medium.

7.3.1.3 Chondrogenic differentiation

Expanded cells were detached by trypsinization (3min) to be further re-differentiated in macro and micro-masses (*sections 4.1 and 4.2*) or in scaffolds (*section 4.3*). For **macromases** (N-TEC/A-TEC and OA-OC aggregates), pellets were generated through centrifugation (1500rpm, 3min) of 250.000 cells *(256)* in non-adherent 96 well plates, and cultured in chondrogenic medium for up to 14 days. **Micromases** were treated as previously described *(254)*. Briefly, about 100 cells were seeded on a microfluidic device with multiple cubic chambers (side of 150 μm) to obtain

microaggregates that were then allowed to condensate at static condition for one day, followed by 7 days of differentiation within the device in serum-free chondrogenic medium (same that for macromases) under continuous perfusion (0.5 µl/h).

Differentiation in scaffolds was performed by seeding the chondrocytes at a density of $4.2x10^6$ cells/cm^3 in a collagen-based sponge (8mm diameter), to be further differentiated during two weeks, according to protocols for clinical grade manufacturing of human nasal chondrocytes-based grafts *(157)*. In all cases, medium change was performed twice a week.

7.3.1.4 Generation of conditioned media

7.3.1.4.1 From osteoarthritic cells/tissues

From ***OA-OC***: Pieces were harvested, and processed as previously described *(193)*, by cutting them into pieces of about $9cm^2$, and individually culturing the fragments for 7d in 8mL of osteogenic medium. From ***OA-Syn***: Isolated cells were seeded on a density of $1x10^5$ cells/ml in CTR media during 3d. At the end of each culture period, supernatants were collected and used as a pool.

7.3.1.4.2 From Nasal/Articular chondrocytes

To apply into OA-OA aggregates, N-TEC and A-TEC were cultured (in scaffolds for application on OA-AC, and in macromases for application in OA-Syn) in CTR medium at a density of $5x10^6$cells/mL. In both cases, supernatants were collected after 72h to be used as conditioned medium (N-TEC_CM/A-TEC_CM).

7.3.1.5 Cell/tissue treatments

7.3.1.5.1 Exposure of N-TEC/A-TEC to OA-simulating conditions

Re-differentiated nasal or articular chondrocytes in the form of TEC, were culture for up to 2 weeks in CTR mediu or different inflammatory conditions: *(i)* ***Low inflammation*** (CTR containing

50pg/mL IL1β, 50pg/mL TNFα,100pg/mL I6); *(ii)* ***High inflammation*** (CTR with 500pg/mL IL1β, 500pg/mL TNFα,1000pg/mL IL6); *(iii)* ***OA-Syn_CM*** (50% CM/ 50% CTR and *(iv)* ***OA-OC_CM*** (30% CM/ 70% CTR). During the exposure time, medium containing the corresponding factors was renewed twice a week and at the end of the culture, TECs were harvested and stored for analysis.

7.3.1.5.2 Exposure of OA-cells and tissues to conditioned media

After chondrogenic differentiation, aggregates of ***OA-AC*** were culture in N-TEC/A-TEC_CM medium during 7d before analysis. ***OA-Syn*** (in monolayer) and ***OA-OC*** (in pieces of 0.25cm^2 aprox), were also cultured in the conditioned medium from N-TEC and A-TEC for up to 14 and 7d before further processing.

7.3.1.6 Generation of N-TEC/OA-OC constructs

7.3.1.6.1 Engineered model

Expanded human NC were labelled with GFP via lentiviral transduction *(280)* and cultured in collagen-based sponges (6 mm diameter) for 4 days in basic medium (5% FBS) containing TGF-β3, AA and insulin. In parallel, OA-OB 3-4.5x10^3/mm^3 were seeded on cylindrical ceramic scaffolds (6 mm diameter, 4 mm high) and cultured in osteogenic medium. At day 4, N-TEC were combined with the bone-cylinders seeded with OA-OB, using fibrin glue. Constructs were incubated for 24 h in basic medium (supplemented with 2% FBS, AA, dexamethasone, glycerophosphate) prior subcutaneous implantation. Identically generated constructs were maintained in parallel in vitro for the same time.

7.3.1.6.2 Explant model

One day prior implantation, a defect of 6mm^2 was created in the cartilage layer of the osteoarthritic osteochondral (OA-OC) tissues by a combination of punching and spooning out the unwanted tissue. Afterwards, the generated N-TEC were also punched to the same diameter than the defect and placed into for further implantation of the combined construct. Uncombined N-TEC and OA-OC plugs were left as controls.

7.3.1.6.3 Ectopic Implantation

Constructs of the respective models in nude mice (Nu/Nu, Charles-River, Sulzfeld, Germany) for 8 weeks as previously described *(281)*. Two to three constructs (combinations or controls) were implanted per animal. Additional OA-OC, N-TEC and OA-OC+N-TEC were kept in culture as in vitro controls.

7.3.1.6.4 Sheep model

7.3.1.6.4.1 Defect creation

OA was induced as described before in caprine models *(282)*, by generation of four full thickness cartilage defects of 4mm in diameter/animal (2 per condyles) in female white Swiss alpine sheep (n = 6) (71.0 ± 4.1 kg; 1.5 - 2.5 years). Defects were left untreated until implantation (for 8 weeks) under free movement and load bearing, with no mechanical support.

7.3.1.6.4.2 Autologous sheep N-TEC in osteoarthritic articular cartilage defects

Sheep NC were isolated (n=6 animals), for generation of sheep N-TEC (sN-TEC) as previously described *(143)*. Briefly, circular biopsies of 6mm in diameter were harvested and kept in transport medium (PBS, 500ng/mL fungizone, 200U/mL PSG) till processing. Once in clean conditions samples were fragmented, enzymatically digested and expanded for two passages. At this stage, cells were GFP-labelled as previously described *(156)* by plating 4x10^{3}cells/cm^2 overnight in basic

medium (2% FBS) to then add a lentiviral suspension (MOI 10) in the presence of polybrene (2µg/mL) during 4 hrs, time after which cells were back to basic medium. Transduction efficiency was inspected either visually or by FACS 24hrs after viral exposure.

Three weeks prior implantation sNC were thawed, let attached to plastic for 24hr and used for chondrogenic differentiation in scaffolds according to protocols for clinical N-TEC manufacturing *(157)*. After that, autologous sN-TEC were implanted into the OA induced defects and left for up to 12 months.

7.3.2 Analytical methods

7.3.2.1 Tissue quality characterization

7.3.2.1.1 Fixation, paraffin embedding and cutting

After culture phase, engineered cartilaginous tissues were stored in formalin overnight at 4°C, and afterwards washed with PBS, dehydrated and embedded in paraffin by means of an automatic immersion device and a tissue embedding station. Embedded tissues were then sectioned (4 to 6µm thickness) and stained for different molecules upon rehydration. For osteochondral tissues, a step of decalcification with EDTA was introduced *(283)* (up to 4 months according to the size) prior paraffin embedding.

7.3.2.1.2 Safranin-O:

Proteoglycans present on the sections were detected with Safranin-O as previously described *(243)*, and counterstaining with fast-green and hematoxylin. Colored sections were permanently mounted for imaging.

7.3.2.1.3 Immuno-detection

Sections were enzymatically digested, with hyaluronidase (2mg/mL, 15 to 30min) and pronase (1mg/mL, 30 to 60min) at 37°C. Then, sections were washed, and goat serum used to block non-

specific epitopes prior antibody exposure overnight. Afterwards, for immunohistochemistry (IHC), matching biotinylated-secondary antibodies were used (2hr at RT) and biotin positive regions detected with alkaline phospathase coloured-substrate-binding complexes. Finally, nuclei were stained with hematoxylin and slides mounted and imaged. For immunofluorescence (IF), sections were treated as for IHC staining until primary antibody exposure. After, a matching secondary antibody fluorochrome-conjugated was used for detection. Nuclei were stained with Dapi (300mM). For micropellets, IF analyses were performed as previously described *(284)*.

7.3.2.1.4 Alu in situ hybridization

Detection of Alu genomic sequences was performed as described before *(143)*. Briefly, hybridization was performed over night at 42°C on paraffin sections using a digoxygenin labelled probe specific for human Alu genomic repeats, and identified with an alkaline phosphatase conjugated antibody.

7.3.2.2 Biochemical characterization

Engineered tissues were digested in 500 µL of protein kinase (1mg/mL) at 56°C during 16hrs and the obtained suspension, processed immediately or stored at -20°C for the subsequent analysis.

7.3.2.2.1 GAG quantification:

Glycosaminoglycans were measured in the digested engineered tissues as previously described *(285)*, by homogenizing 20 µL of the sample in 1mL of dimethylmethylene blue (DMMB) to allow cluster formation. Then, samples were centrifuged to recover the GAG-DMMB complexes as pellets, to be further dissolved and spectrophotometrically quantified. GAG concentration on the assessed TECs were determined through interpolation of a typical Em/Concentration standard curve prepared with Chondroitin Sulphate.

7.3.2.2.2 DNA quantification:

DNA was quantified in digested tissues with CyQuant assay according to manufacturer instructions. DNA was then measured spectrophotometrically. Concentration on targeted samples were determined by interpolation of an Emission/Concentration standard curve prepared with bacteriophage λ DNA.

7.3.2.3 Image acquisition and processing

Bright field and fluorescent images were acquired with an inverted microscope. No additional processing was done on bright field captures, while fluorescent images further processed with the Fiji image processing package in order to compose RGB pictures.

7.3.2.3.1 Histological evaluation of engineered-tissue quality

Quality of the obtained tissues was assessed visually on Safranin-O stained sections using the Bern Score *(243)*. Each sample was graded from 0 to 3 arbitrary units, in terms of (A) uniformity and intensity of the staining, (B) distance between cells/amount of matrix produced and (C) cell morphology. Three independent observers judged all parameters, and the grades averaged for a 0 to 9 global qualification; being 0 the lowest and 9 the highest cartilaginous quality.

7.3.2.3.2 Quantification of Safranin-O staining

Indirect quantification of GAG content was performed by measuring of the optical density (OD) of Safranin-O by deconvolution of the RGB images and subtraction of the OD for fast green and hematoxylin, assuming stoichiometric staining *(286)*. The intensity range was defined from min (= darkest pixel) to max (=the empty area of the image) and calculated for the whole image as an average from all pixels, normalized by the max intensity. Values where then correlated with visually scored samples for the (A) parameter in the BernScore (previous section) to set the measurments between 0 and 3, according to the stablished scale. Analysis were performed with Fiji.

7.3.2.3.3 GFP quantification for detection of NC in sheep samples

Total cells (DAPI) and GFP-positive NC were quantified through bright spot counting in the corresponding channels (General Analysis tool, NIS-Elements software). Percentage of GFP-positive cells out of all DAPI positive cells were calculated in the graft area (defined as the presumed graft area - that may include up to 100 µm of adjacent cartilage since the definite borders were not always clearly definable -) or in the interzone (defined as the whole cartilaginous layer without the graft area). Both graft and interzone included approximately 50 µm of the underneath calcified cartilage/subchondral bone. Quantification was performed for 2 individual section of each construct.

7.3.2.4 Gene expression analysis

For cells and macromases, stored samples (-80°C) were thawed and sonicated (in the case of engineered tissues) prior total RNA isolation and purification with a miniprep Kit, according to manufacturer instructions. In the microfluidic device, microaggregates were perfused with PBS for 10 minutes (100 µl/h), and subsequently with lysis buffer for 10 minutes (300 µl/h) to be recovered downstream in a collection tube before preparing the miniprep.

For qRT-PCR, the extracted RNA was retrotranscribed to cDNA with SuperScript transcriptase following supplier protocol, and the product of the reactions used for quantitative PCR to determine expression levels of *COL2, COL1, ACAN, MMP13, MMP3, ADAMTS5, IL8 and IL6* and *GAPDH* as housekeeping gene. Specific primers are reported in Table 18. Upon amplification, C_T values were analyzed with the $2^{-\Delta\Delta CT}$ method *(287)*.

7.3.2.5 Protein quantification

For ***in vitro experiments***, supernatants from the different conditions were collected at the specific time points and stored at -80°C until processing. Samples were then thawed and centrifuged at

16.000xg during 4 min to remove particulates and then used for the different assays. IL1β, IL6, IL7, IL8, IL12, IL17, Eotaxin, GCSF, IFN, IP10, MCP1, MIP1α, MIP1β, RANTES, TNFα, IL4, IL10, IL13, IL1Ra and MMP3 were quantified by Luminex; MMP13, ADAMTS4 and ADAMTS5 by ELISA.

For ***sheep synovial fluids***, cytokine concentrations were determined with sheep specific kits for IL1β, IL6, IL8/CXCL8, IL10, TNFα, IL4, IL7, IL13, MMP13 and - due to a lack of sheep specific ELISAs -, with bovine assays for MMP1, MMP3 and ADAMTS5.

7.3.2.6 Statistical analyses

All data are presented as mean values ±SD (standard deviation). For each donor and experimental group, technical duplicates or triplicates were performed. Multiple comparisons were assessed by Kruskal Wallis and pairs by Mann Whitney-U test, when this last comparison was used as post hoc correction of the *P* value with the Bonferroni method was done. *P* values < 0.05 were considered significant. Sample size and specific analysis are indicated on each figure. All comparisons were done with the SPSS statistical package.

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Appendix

List of Abbreviations

A-TEC	tissue engineered cartilage derived from articular chondrocytes
A-TEC_CM	medium conditioned by A-TEC_CM
AA	ascorbic acid
AC	articular chondrocytes
ACAN	encoding gene for aggrecan
ACI	autologous chondrocyte implantation
ADAMTS a	disintegrin and metalloproteinases with thrombospondin motif
ASC	adult stem cell/progenitors
BMP	bone morphogenic proteins
COL2/1	encoding gene for type II/I collagen
COMP	cartilage oligomeric protein
DMD	disease modifying drugs
ECM	extracellular matrix
EGF	epidermal growth factor
ESC	embryonic stem cells
FGF	fibroblastic growth factor
GAG	glycosaminoglycans
GFP	green fluorescent protein
HA	hyaluronic acid
HOX	subset of homeobox genes
IGF	insulin growth factor
iPSC	induced pluripotent stem cells

ITS	insulin-transferrin-selenium
MACI	matrix-based autologous chondrocyte implantation
MMP	matrix metalloproteinases
MSC	mesenchymal stromal cells
N-TEC	tissue engineered cartilage derived from articular chondrocytes
N-TEC_CM	medium conditioned by N-TEC
NC	nasal chondrocytes
NSAIDs	nonsteroidal anti-inflammatory drugs
OA	osteoarthritis
OA-OC_CM	medium conditioned by osteoarthritic osteochondral tissues
OA-Syn_CM	medium conditioned by osteoarthritic synovial cells
OAT	osteochondral allograft transplantation
OB	osteoblast
OC	osteochondral tissus/explants
OCT	osteochondral autograft transplantation
PG	proteoglycans
RANTES	Regulated upon Activation, Normal T cell Expressed, and Secreted cytokine
Syn	synovial cells
TE	tissue engineering
TGF-β	transforming growth factor beta
TNFsR	tumor necrosis factor soluble receptor
TNF*α*	tumor necrosis factor alpha
TSP	thrombospondin
TSPSC	tissue specific progenitor stem cells
VEGF	vascular endothelial growth factor
Wnt	wingless/integrated-1 protein

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