UiT
THE ARCTIC UNIVERSITY OF NORWAY

Faculty of Health Sciences - Department of Clinical Medicine

Biology of signalling receptors in human articular chondrocytes

Implications for chondrogenesis and cartilage repair

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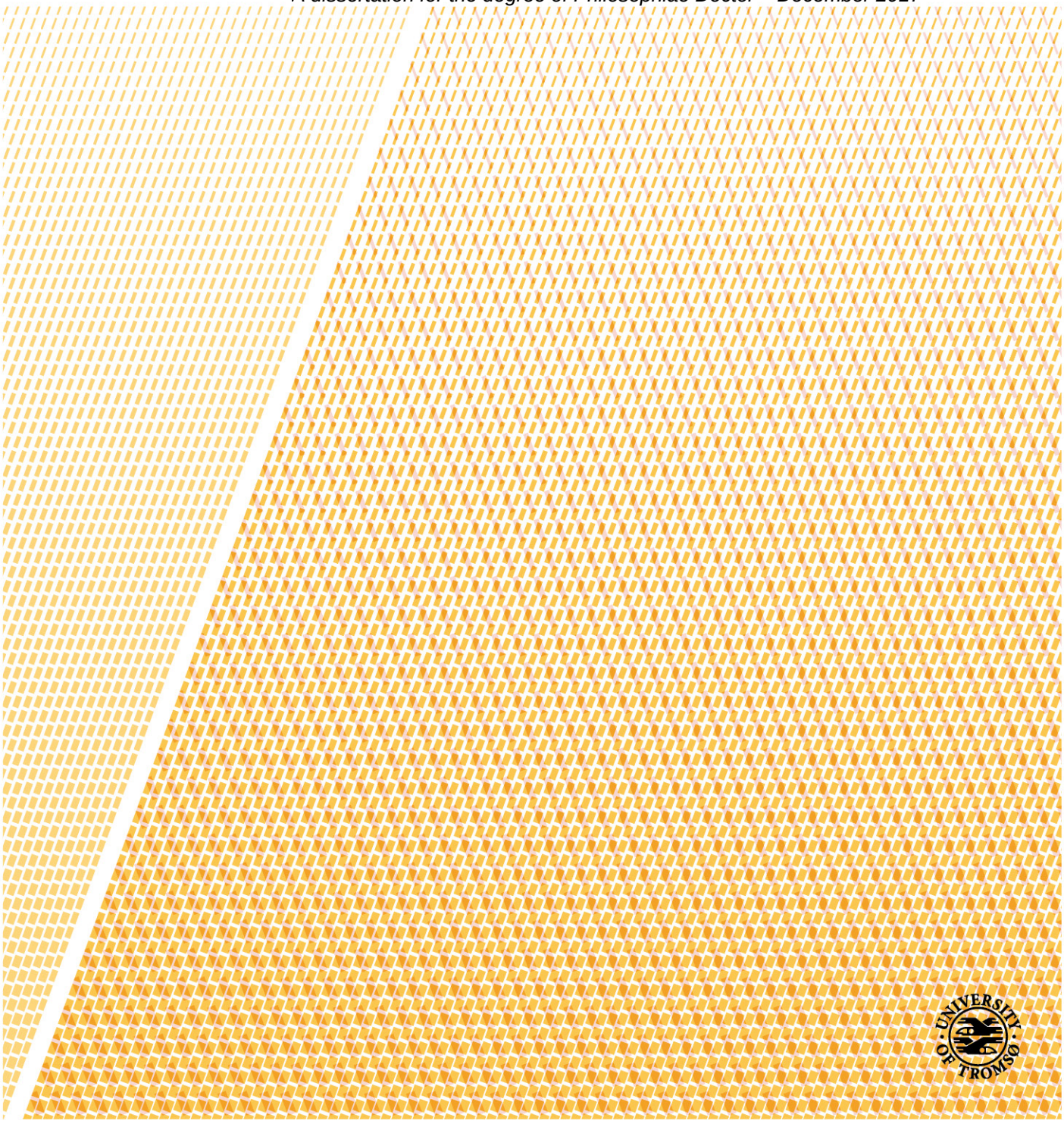
Ann Kristin Hansen

A dissertation for the degree of Philosophiae Doctor – December 2017



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Were there none who were discontented with what they have, the world would never reach anything better.

Florence Nightingale

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Acknowledgements

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English summary

Osteoarthritis (OA) is characterised by gradual destruction of articular cartilage and leads to painful and dysfunctional knee-, hip- or hand-joints. With the increasing life expectancy and obesity, the prevalence is expected to rise. In the younger population, joint pain and disability can result from local cartilage defects resulting from injury or disease. After the introduction of autologous chondrocyte implantation (ACI) as a treatment option for localised cartilage defects in the late 80's, cell-based repair techniques have been extensively explored through clinical trials aiming to improve long-term clinical outcomes. However, the role of ACI remains that of postponing joint replacement in patients with localised defects, and despite persistent research on cartilage repair strategies, there is no available treatment to halt or reverse the degenerative process of OA once initiated. The focus of this thesis has thus been to gather new basic knowledge on cartilage functions in the context of cell signalling receptors.

In the first study, we have explored the effects of the powerful inflammatory mediator **leukotriene B_4** (LTB_4) on human articular chondrocytes based on studies indicating that this mediator could hold a key role in inflammatory joint diseases. When cyclooxygenase (COX) inhibitors are prescribed to reduce inflammation and pain e.g. in patients with OA, there is a shunting from the prostaglandin- to the leukotriene axis with subsequent upregulation of LTB_4. We demonstrated that chondrocytes express both the high-affinity (BLT1) and the low-affinity (BLT2) LTB_4 receptors by immunolabelling and gene expression. By Western blot, we showed that the high-affinity BLT1 receptor is active. Upon stimulation of chondrocyte cultures by the ligand, we found no effect on biological functions such as release of inflammatory mediators, proliferation, cartilage gene expression or matrix formation. The overall results suggest that the leukotriene axis is not very active in cartilage, and that the role seen in other inflammatory diseases is probably linked to the ability of LTB_4 to recruit neutrophils, a mechanism that is less prominent in osteoarthritis pathology.

In the second paper, we investigated the influence of **the active hormonal form of vitamin D, $1\alpha,25(OH)_2D_3$**, on chondrocyte functions and evaluated potential modulating effects on inflammation as suggested by clinical studies showing improved pain scores after vitamin D supplementation. By immunolabelling and gene-expression, we found that the expression of vitamin D receptor (VDR) in native cartilage is elusive, but that receptor expression increase upon dedifferentiation and during inflammatory conditions. We also demonstrated that 1α-hydroxylase, the enzyme catalysing the conversion of $25(OH)D_3$ to $1\alpha,25(OH)_2D_3$, is expressed in cartilage and that the expression persists through cellular dedifferentiation and redifferentiation. In monolayer cultures the $25(OH)D_3$ was converted to $1\alpha,25(OH)_2D_3$ in a dose dependent matter, and exposing chondrocytes to both $25(OH)D_3$ and $1\alpha,25(OH)_2D$ increased their proliferation rate. The proteoglycan genes ACAN and VCAN displayed an inverse expression pattern, and matrix production was diminished in chondrocytes treated with $25(OH)D_3$ or $1\alpha,25(OH)_2D_3$. The results imply that cartilage can contribute to the increased level of $1\alpha,25(OH)_2D_3$ seen in synovial fluid of OA patients, but $25(OH)D_3$ or $1\alpha,25(OH)_2D_3$ may only exert effects on chondrocytes upon dedifferentiation or during inflammatory conditions.

The third paper aimed at identifying biomarkers of intrinsic chondrogenic potential in chondrocyte cultures established from 17 donors undergoing ACI treatment. Patient-derived chondrocytes cultures were grouped according to their chondrogenic abilities in scaffold-free 3D cultures, as evaluated by the Bern score. The groupwise expression of cell-surface molecules including integrins, cell adhesion

molecules and growth factor receptors were measured using flow cytometry or gene expression. The gene expression of TGF-β receptor 3 was inversely related to chondrogenic potential, while all other molecules tested had a uniform expression pattern among all donors. A global proteomic profiling of cell-associated proteins using tandem-mass-tag technology pointed at prolyl 4-hydroxylase, a pivotal enzyme in collagen triple helix formation, as a biomarker potentially linked to chondrogenic potential.

List of papers

Paper I:
Hansen, A. K., Indrevik, J.-T., Figenschau, Y., Martinez-Zubiaurre, I., & Sveinbjörnsson, B. (2015). Human articular chondrocytes express functional leukotriene B 4 receptors. Journal of Anatomy, 226(3), 268–277. https://doi.org/10.1111/joa.12275
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Paper II:
Hansen, A. K., Figenschau, Y., & Martinez-Zubiaurre, I. (2017). Co-expression of 1α-hydroxylase and vitamin D receptor in human articular chondrocytes. Submitted.

Paper III:
Islam, A., Hansen, A. K., Elvenes, J., & Martinez-Zubiaurre, I. (2017). Uniform expression of cell-cell and cell-matrix receptors in patient-derived chondrocyte cultures with divergent *in vitro* chondrogenic capacity. Manuscript.

List of abbreviations

$1\alpha,25(OH)_2D_3$	1α,25-dihydroxy-vitamin D_3
$25(OH)D_3$	25-hydroxy vitamin D_3
5-LOX	5-lipoxygenase
ACI	Autologous chondrocyte implantation
ADAMTS	A disintegrin and metalloprotease with thrombospondin motifs
ACs	Articular chondrocytes
ALCAM	Activated-leukocyte cell adhesion molecule, CD166
BLT1 and BLT2	Leukotriene B_4 receptor type 1 and 2
BMP	Bone morphogenetic protein
CAM	Cell adhesion molecule
CD44	Hyaluronan receptor
CD271	Low-affinity nerve growth factor receptor, LNGFR
COL1A1	Collagen type 1
COL2A1	Collagen type 2
COX	Cyclooxygenase
CYP27B1	Gene encoding 1α-hydroxylase
ECM	Extracellular matrix
FAK	Focal adhesion kinase
GAGs	Glycosaminoglycans
GOI	Gene of interest
HA	Hyaluronic acid
ICAM-1	Intercellular adhesion molecule-1, CD54
IL-1β	Interleukin-1β
IL-6	Interleukin-6
IL-8	Interleukin-8
LTB_4	Leukotriene B_4
MAPK	Mitogen-activated protein kinase
MMP	Matrix metalloproteinase
MSC	Mesenchymal stem cell
NO	Nitric oxide
NSAID	Non-steroidal anti-inflammatory drug
OA	Osteoarthritis
PCR	Polymerase chain reaction
PGE_2	Prostaglandin E_2
RA	Rheumatoid arthritis
RT	Reverse transcriptase
SMAD	SMA (small body size gene and protein in C. Elegans) + MAD (mothers against decapentaplegic, protein in Drosophila) = SMAD
TGF-β	Transforming growth factor-β
TGFBR	Transforming growth factor-β receptor
TMT	Tandem-mass-tag
VDR	Vitamin D receptor
VCAM-1	Vascular cell adhesion molecule -1
qPCR	Quantitative real-time polymerase chain reaction

Definitions of key concepts

Bern score
The Bern score is a visual histological scoring and accounts for uniformity and intensity of matrix staining, cell density/ECM ratio and cellular morphology.

Cell adhesion molecules
Cell-surface molecules, usually glycoproteins, which mediate cell-cell and cell-matrix interactions and thus maintain tissue integration.

Cell differentiation
Progressive restriction of the developmental potential and increasing specialization of function that leads to the formation of specialised cells, tissues, and organs.

Cell dedifferentiation
A reverse developmental process in which terminally differentiated cells with specialised functions revert to a less differentiated stage within their own cell lineage. The loss of specialisation in form of function.

Cell redifferentiation
The process where dedifferentiated cells return to their original specialised form.

Chondrogenesis
The formation of cartilage proceeds in two main stages: condensation and differentiation. During condensation, the cell-cell adhesion molecules N-cadherin and N-CAM are key players, while in the differentiation stage the cell-matrix interactions are more important.

Extracellular matrix
The extracellular matrix of cartilage consists of a network of collagens that provide tensile strength and proteoglycans responsible for osmotic swelling and the elastic properties, serving as a scaffold for the chondrocytes and as a reservoir for growth factors and cytokines.

Integrins
Transmembrane glycoproteins built from one α and one β subunit that interact with extracellular matrix outside the cell and the cytoskeleton inside the cell. These receptors facilitate both cell-matrix and cell-cell cross talk.

Monolayer culture
Cells that are enzymatically relieved from the surrounding matrix and allowed to adhere to and expand in plastic flasks or vessels in a single layer fashion, most commonly composed of a single cell type.

Spheroid culture
Cells are collected in spheroids by gravity (hanging-drop method) or centrifugation (pellet method), and propagated in chondrogenic medium. In the works presented in this thesis, the method is used to assess chondrogenic potential of chondrocytes.

Suspension culture
Cell cultures established by gentle enzymatic digestion of tissue and incubation of cells in ultra-low binding culture vessels to prevent cell attachment and dedifferentiation.

1 Introduction

1.1 Articular cartilage

Hyaline cartilage is an avascular, aneural and alymphatic tissue covering the ends of long bones facilitating a frictionless movement and absorption of forces in the diarthrodial joint. The thickness of cartilage is related to the congruency of the joint, ranging from 1.2 mm in the congruent ankle joint to 2.17 mm in the incongruent knee joint of adults (1), while in adolescents knee joints the thickness range up to 4 mm (2). Cartilage consists of only 3-4 % cells, while the bulk of the tissue is the surrounding matrix made up of collagen type II and glycosaminoglycan that provide structural architecture and captures water molecules. The tissue is spatially organised in a superficial, middle, deep and calcified zone. While most reports have named the chondrocyte as the sole cell type in cartilage, newer publications have reported progenitor cells residing in the superficial layer (3). The zonal organisation of the matrix facilitates the highly specialised mechanical properties of hyaline cartilage. The superficial zone is designed to handle the sheer forces of the moving joint with flattened chondrocytes and fibrils arranged parallel to the joint surface. The compressive forces are handled by the obliquely organised middle layer and particularly the deep layer where the cells are arranged in columns and the fibrils run perpendicular to the joint line (4). The tidemark is the basophilic line on histological sections separating the hyaline cartilage from calcified cartilage, while the cement line separates the calcified cartilage from the subchondral bone plate (5). The zonal organization is reflected in the chondrocytes exhibiting different phenotypes in the superficial, middle, deep and calcified zones (6).

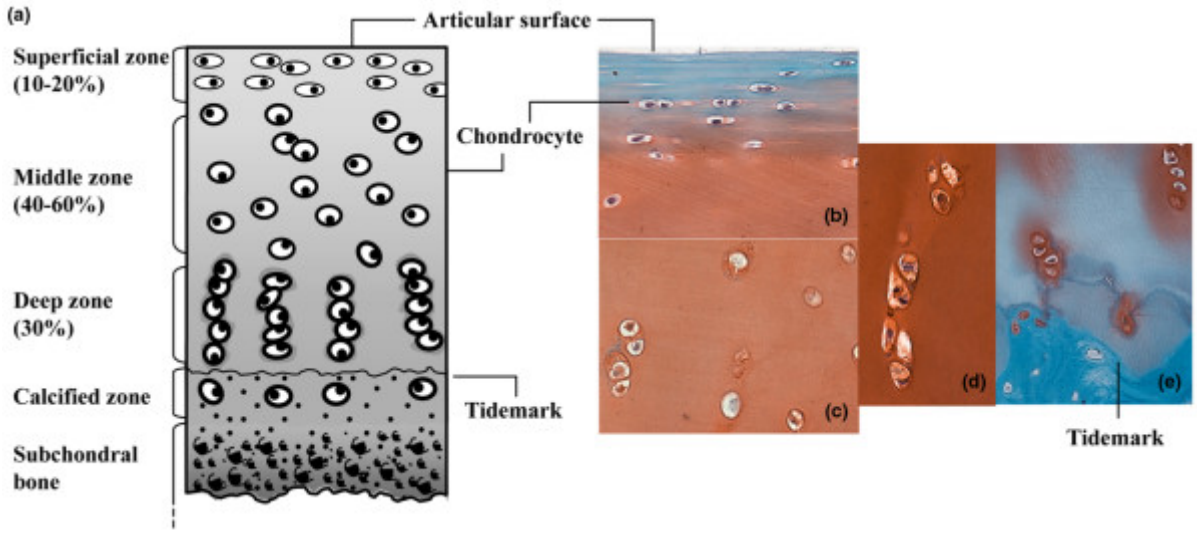

Figure 1 Zonal architecture of cartilage (a), micrograph of Safranin O stained sections of cartilage of the superficial zone (b), middle zone (c), deep zone (d) and calcified zone (e). The image is reprinted from Grogan et al. 2009 (7).

In addition to the zonal organisation, the cartilage can also by described by pericellular, territorial and interterritorial regions. One or a few chondrocytes are organised in chondrons where they are spaced by pericellular matrix dominated by proteoglycans, collagen type VI and membrane receptors called integrins that anchor the chondrocyte to the surrounding territorial matrix. The interterritorial matrix space the chondrons and consists mainly of collagen type II and proteoglycans (8). The pericellular

matrix surrounds the chondrocyte by 2 µm, the territorial matrix occupies the area 2-5 µm from the chondrocyte and the interterritorial matrix is spaced more than 5 µm from the chondrocyte (9). The matrix captures 65-80 % water in the tissue, leaving the dry weight of the extracellular matrix at around 20-35 %. The matrix constituents have a long half-life where collagen type II for instance, has a half-life of >117 years (10).

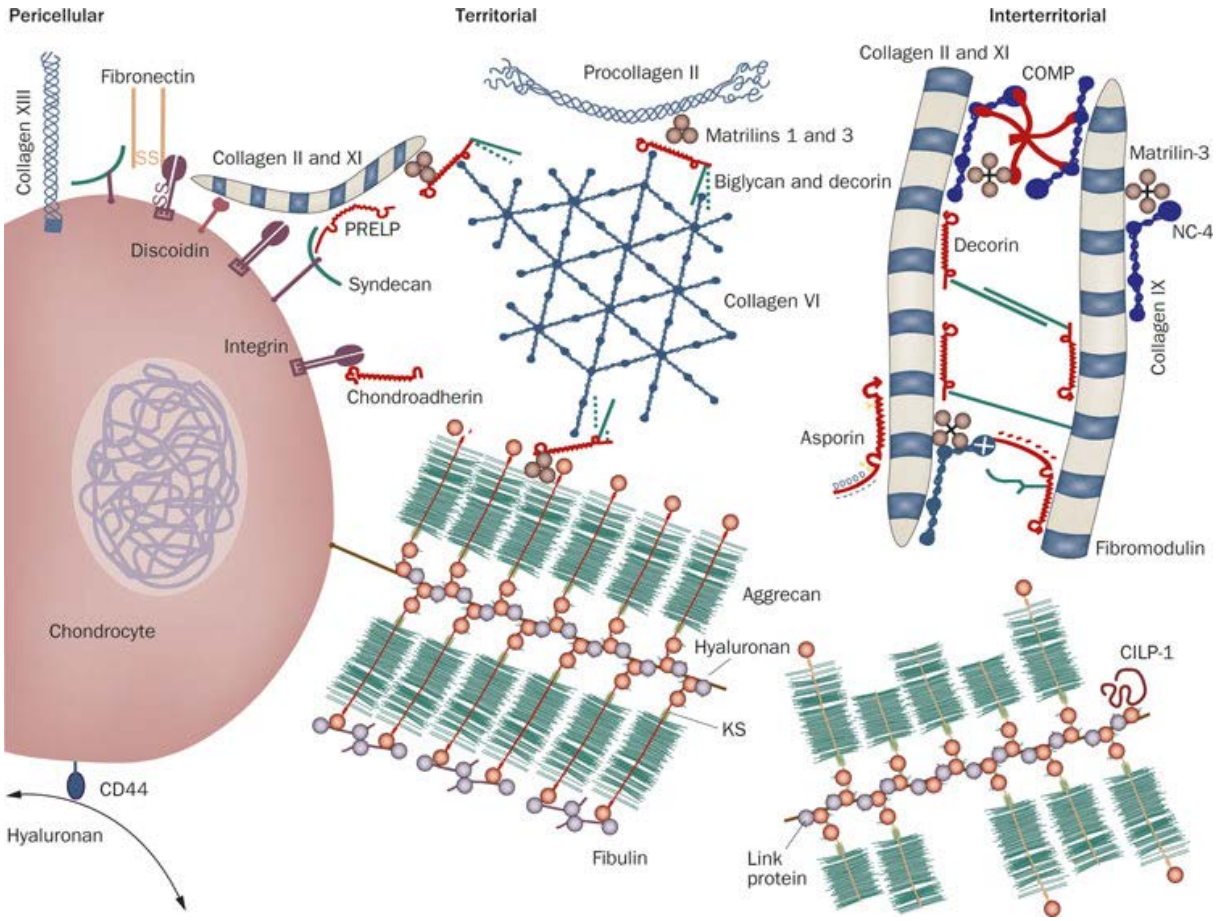

Figure 2 Cartilage territorial organization. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Rheumatology (11)*, copyright 2011*

Due to its shock absorbing and articulating functions, articular cartilage cannot afford blood, lymph or nerve supply. The chondrocyte is well adapted to hypoxia and low O_2 tension is necessary for the chondrocyte to retain its phenotype and support the matrix homeostasis (12). The avascular and alymphatic nature of cartilage leaves nutrients exchange to diffusion from synovial fluid and to a lesser degree the subchondral bone. The oxygen tension forms a gradient from around 10 % in the superficial zone down to 1 % in the deep zone. The oxygen consumption of the chondrocyte is about 2-5 % of that of the liver or kidney cell. Chondrocytes produce energy by glycolysis, and the resulting lactic acid molecules are ionised and removed from the cell. The deep zone is thus characterised by low O2, low glucose and low pH, and the lactate level in synovial fluid is 5-8 nM compared to 1 nM in serum (13). Importantly, the avascular nature of cartilage implies that the tissue lacks the competence to heal once injured or diseased.

In the ageing cartilage, the tidemark that separates calcified and uncalcified cartilage moves towards the cartilage surface. The uncalcified zone gets thinner with advancing age as the lower calcified zone

is replaced by bone. Thus, the thinning of the cartilage is not only a result of continuous wear and tear, but also a result of calcified cartilage being replaced by bone (14).

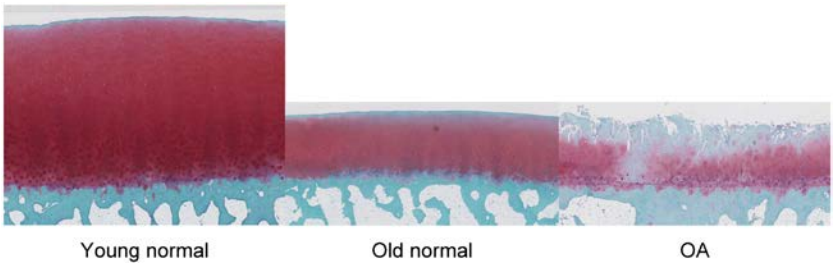

Figure 3 Thinning of cartilage. Safranin O stained sections of human femoral condyles from 40-year-old (left), 76-year-old (middle) and 88-year-old OA (right) donors. Reproduced from Lotz et al. 2012 (15)*, with permission from Elsevier.*

1.2 Osteoarthritis

The Osteoarthritis Research Society International have suggested the following definition of osteoarthritis in their 2015 review (16):

"Osteoarthritis is a disorder involving movable joints characterised by cell stress and extracellular matrix degradation initiated by micro- and macro-injury that activates maladaptive repair responses including pro-inflammatory pathways of innate immunity. The disease manifests first as a molecular derangement (abnormal joint tissue metabolism) followed by anatomic, and/or physiologic derangements (characterised by cartilage degradation, bone remodelling, osteophyte formation, joint inflammation and loss of normal joint function), that can culminate in illness."

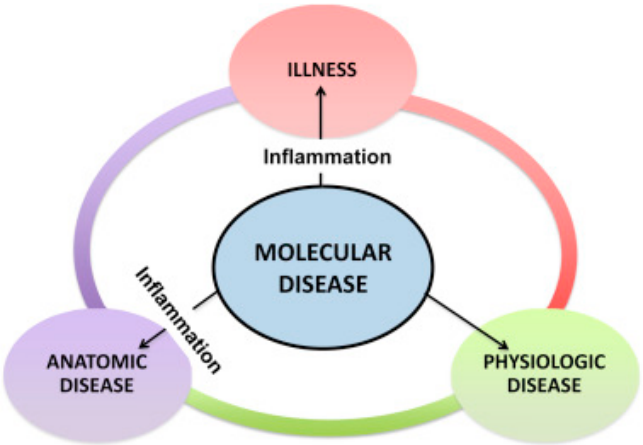

Figure 4 OARSI definition of osteoarthritis. The molecular disease represent early asymptomatic OA that precedes physiological and anatomic aspects by years. This makes it challenging to detect the disease at an early stage, but also represents a window of opportunity to discover new early markers. Figure reprinted from Mobasheri et a 2017 (17)*.*

In the clinical setting, the diagnosis of OA is based on the patient's history of pain and disability, supported by radiological evaluation by x-ray, MRI or ultrasound. The radiological findings on x-ray can be graded according to the Kellgren and Lawrence score that levels OA deterioration from none to severe (18). The prevalence of this debilitating disease was reported to 12.8 % in Norwegian study of OA in the hip-, knee- and hand joints. The prevalence increase with advancing age and for people in

their fifties and sixties the prevalence of knee OA alone was 12.2 % (19). Radiological confirmed OA of the knee was estimated to 3.8 % on a global scale in data from 2010 including everyone over the age of thirty (20). The drawback of resting solely on radiologic assessment is that the association between radiological findings and clinical presentation is weak (21). With the rise in life expectancy, along with an increase in other risk factors such as obesity, joint trauma and metabolic disorders, OA prevalence has been predicted to rise up to 50 % over the next 20 years (22).

OA has been considered a "wear and tear" initiated degeneration of the cartilage, but the condition involves the entire joint including subchondral bone, meniscus, ligaments, tendons, synovium and muscles (21,23). The idea of an inflammatory component is not new, it was introduced in Ehrlich's report on hand OA from 1975 (24). With recent advances, it has become clear that molecular inflammation is a contributor, and that tissue degeneration and inflammation interact in a vicious circle once initiated. Pro-inflammatory substances such as nitric oxide (NO), prostaglandin E_2 (PGE_2), interleukin-1β (IL-1β), IL-6 and IL-8 are upregulated in OA and induce a molecular inflammation that is not necessarily clinically visual, but leads to an interruption of cartilage homeostasis and progression of articular degeneration (25,26). Since most cases of OA are idiopathic and symptoms present gradually, the inception is very difficult to determine (27). As opposed to rheumatoid arthritis where the inflammation is orchestrated by migratory inflammatory cells, the inflammation in OA is triggered by resident cells in cartilage or synovium (28). Whether the inflammatory cascades is initiated by chondrocytes or synoviocytes, or even subchondral bone, is thus a matter of debate. Early signs of degradation can be detected using modern imaging and arthroscopy technologies, but it is likely that the cellular and molecular changes in OA start long before damage is clinically detectable.

OA may have miscellaneous origins and may progress differently in different individuals. During recent years, new classifications for OA have been proposed based on different phenotypes. In the end, all phenotypes converge in a common pathway leading to painful, stiff and poor-functioning joints.

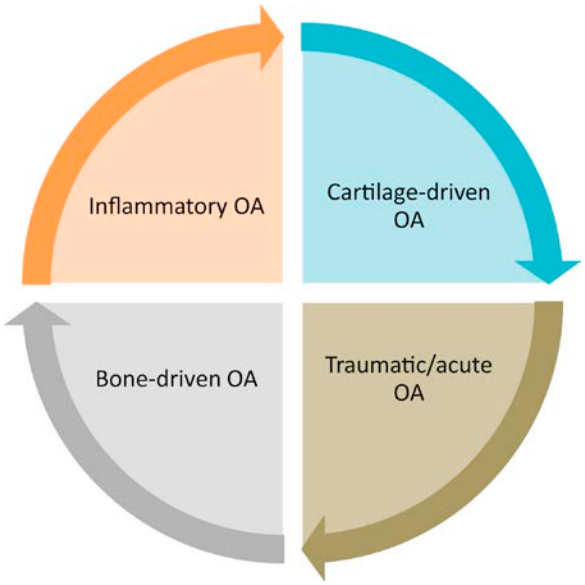

Figure 5 Osteoarthritis phenotypes. Osteoarthritis is a heterogeneous disease originating from different phenotypes. Some phenotypes may be more responsive to pharmacological treatment, while other will be less so, enabling patient stratified treatment. Figure reprinted from Mobasheri et al. 2017 (17).

In the initial stages of OA, proliferative chondrocytes form clusters in order to adjust to the changing microenvironments (29). In normal cartilage, aggrecan is found only in an aggregated configuration of about 100 aggrecan molecules per hyaluronan molecule. In OA, aggrecan occur in a nonaggregated form and the levels decrease, which alters the mechanical compliance of the matrix. Collagen production is upregulated, but switches to production of collagen type I instead of collagen type II. Collagen type I builds shorter collagen fibrils and this makes the cartilage less able to store elastic energy and thus susceptible to fibrillation and fissure formation (30). Progression of OA is characterised by elevated levels of IL-1β and TNF-α which increase the expression of cartilage degrading matrix metalloproteinases (MMPs, i.e. MMP1 and MMP13) and aggrecanases (ADAMTS4, ADAMTS5). Chondrogenic growth- and transcription factors including transforming growth factor-β (TGF-β), sex determining region Y-box 9 (SOX9), insulin-like growth factor (IGF) and connective tissue growth factor (CTGF) are downregulated, prompting a suppression of anabolic activity. The skewed balance between anabolic and catabolic mechanisms in the matrix drastically change the mechanical properties. Initially the extracellular matrix softens due to looser collagen networks allowing the aggrecan to swell (31). As OA progresses, the level of hydrating aggrecan decrease, collagen fibrils stiffen and subsequently more load is transmitted to the underlying subchondral bone. These mechanical changes triggers an advancement of the subchondral bone towards the cartilage surface contributing to cartilage thinning; but also bone marrow oedema, subchondral cysts and periarticular osteophytes. The altered stiffness of the matrix pushes dedifferentiation of the chondrocyte towards a fibrochondrocytic phenotype (30). All evidence taken together suggests that inflammation could have a pivotal role in OA pathogenesis and that developing therapeutic agents targeting inflammation may be a way of preventing or reducing the progression of the disease (32).

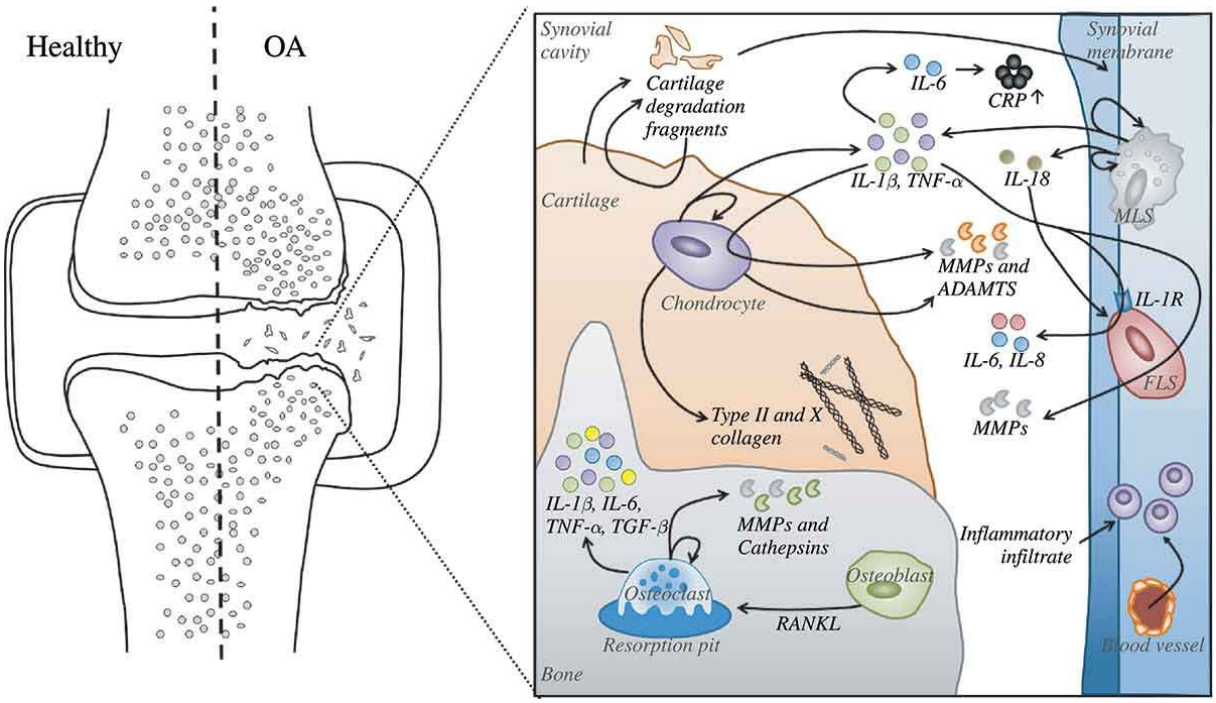

Figure 6 Cells, proteases and proteins involved in cartilage degradation. Figure reproduced from Siebuhr et al. 2016 (33).

1.3 Cartilage injuries

Healthy cartilage can take loads up to 25 MPa, but exceeding this limit cause chondrocyte death and cartilage fissure (34). Three different levels of damage are described with different healing potentials accordingly: (1) damage to cells and matrix without visible damage to joint surface, (2) visible mechanical disruption limited to cartilage, (3) intra-articular fractures. Partial cartilage injuries heal poorly, while intra-articular fracture heal via an initial fibrin clot to a final composition intermediate between hyaline and fibrocartilage (34). Traumatic cartilage injuries are usually the consequence of major knee traumas like anterior cruciate ligament or multiple ligament injures, but repetitive minor trauma could also be the aggressor. **Osteochondritis dissecans** is a joint disorder that is characterised by changes in the subchondral bone and articular cartilage including softening, swelling, early separation, partial detachment or complete osteochondral separation with a loose body. Although the condition has been known for almost 200 years the aetiology is still elusive. Trauma and repetitive micro-trauma has become accepted as a possible cause since the incidence is high among athletes (35). **Primary osteonecrosis** stems from impaired perfusion of the subchondral bone leading to an initial collapse of the bone and secondary deterioration of the overlying cartilage, while a secondary form is observed after the use of corticosteroids or abuse of alcohol (36). Patients who sustain a severe knee injury have about 50 % chance of developing posttraumatic OA, and these patients account for 12 % of all OA cases (27). The natural history of cartilage injuries is not completely unravelled and many patients achieve favourable long-term clinical outcomes after localised cartilage injuries left untreated (37). The patients that do develop painful and dysfunctional joints after injury represent a treatment challenge; the so called: “young patients with old knees” (38).

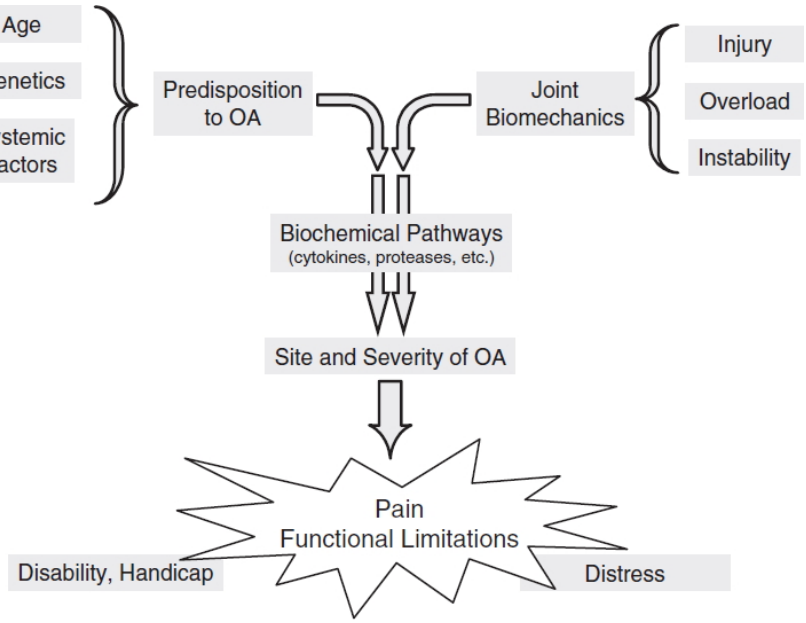

Figure 7 Endogenous and environmental factors contributing to OA initiation and progression. The combination of these factors result in different OA phenotypes. The figure is reproduced from Lohmander et al. 2007 (38).

1.4 Cartilage repair techniques

A number of clinical strategies to repair cartilage damage have been proposed and tested. Modes include pharmacological and surgical techniques (36). The **autologous chondrocyte implantation (ACI)** procedure was first described by Brittberg et al. in 1994 (39), and involves a two-step procedure starting with arthroscopic evaluation of the injured joint and harvest of a cartilage biopsy from a healthy, non-weight bearing area. The biopsy is minced, enzymatically digested and serially expanded in monolayer culture vessels until sufficient number of cells are generated for implantation via a mini-arthrotomy. At the time of chondrocyte implantation, the defect is stabilised by removing any damaged tissue from the defect. A periosteum flap is sutured and glued to the defect and the cell suspension is injected under the flap. The method has since evolved by replacing the periosteum with a collagen membrane, which has alleviated the problem of hypertrophy of the repair tissue encountered in the first generation procedure. The expansion of chondrocytes in monolayer cultures is however flawed by the **dedifferentiation of chondrocytes** into a fibrocartilage phenotype that produce collagen type I instead of collagen type II. Implantation of irreversibly dedifferentiated chondrocytes may be why some patients have worse outcomes than others (40), and identifying tools that enables selection of patients or chondrocytes with properties that favour a good outcome is of great interest in order to improve the technique. Implantation of characterised chondrocytes displaying a chondrocyte-phenotype was introduced to improve hyaline cartilage formation (41). This procedure has evolved into seeding the cells in 3D membrane scaffolds to facilitate the retention of chondrocyte phenotype before implantation (matrix assisted chondrocyte implantation, MACI).

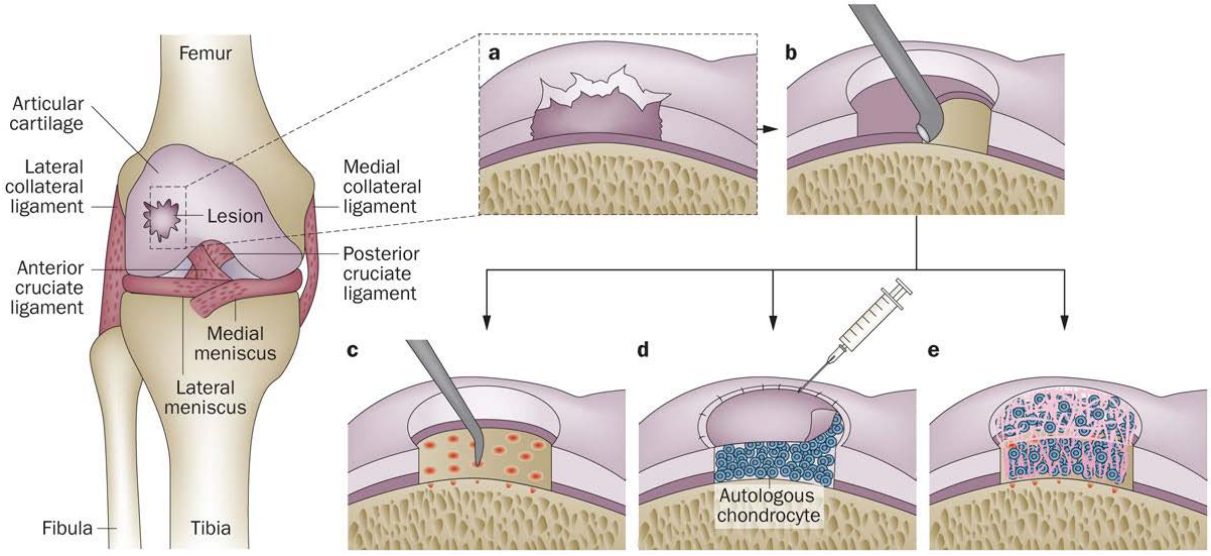

Figure 8 Cartilage regeneration techniques. a) Cartilage lesion, b) debridement of lesion, c) microfracture, d) ACI, e) MACI. Figure reproduced from Makris et al. 2015 (42).

Another treatment option not subjected to ex-vivo expansion of cells is **microfracture**, a technique introduced by Steadman and Rodrigo in 1994 (43) based on the initial report by Pridie in 1959 (44). Concerns about the quality and durability of the repair tissue derived from this method has been posted (45). Autologous matrix-induced chondrogenesis (AMIC), is a refinement of the microfracture technique where a collagen type I/III matrix is added to improve mechanical stability and thus the quality of the repair tissue (46). Studies comparing the clinical outcome of AMIC compared to other methods are ongoing.

Since the advent of ACI, a plethora of methods to regenerate cartilage has been investigated. Broadly, the approaches can be divided into cell-based methods, scaffold based methods and tissue-engineered grafts (47). Cell-based methods explore the use of chondrocytes, mesenchymal stem cells, embryonic stem cells and induced pluripotent stem cells, and rely on replicating the precartilaginous mesenchymal condensation. Mesenchymal stem cells can be harvested from bone marrow, synovial tissue, the infrapatellar fat pad, subcutaneous fat or muscle, thus eliminating the donor site morbidity associated with harvesting cartilage. Cells seeded in protein-, polysaccharide- and synthetic scaffolds providing a 3D environment that promotes chondrogenic differentiation have been tested in clinical studies (48). The optimal scaffold should have mechanical properties matching the existing cartilage, ability to integrate with adjacent cartilage and sufficient durability. Tissue engineering aims to build cartilage constructs from combinations of cells and scaffolds or through scaffold-free approaches. Mechanical stimulation, oxygen tension and 3D environment are used to induce chondrocyte differentiation and matrix production. In addition, biological factors like TGF-β and other growth factors can be combined with any of the above methods in order to induce chondrogenesis. Yet a different approach is the mosaicplasty where cartilage cylinders are harvested from a non-weight bearing part of the joint and transplanted into the injured site, albeit this method is hampered by donor-site morbidity.

1.5 The chondrocyte

The chondrocyte communicates with the surrounding matrix via membrane receptors and organelles like cilia and caveola (49). The normal chondrocyte has a low turnover, replacing resilient cartilage matrix proteins. Once the cartilage is injured or OA is induced the chondrocyte metabolism increase and the balance between synthesis and degradation of matrix protein is disrupted. Degradation products interact with cell-surface receptors and induce an increased production of matrix-degrading proteinases (50). The collagen expression pattern shifts toward type III, VI, X and a chondroprogenitor splice variant of collagen type II (type IIA) (51). This central role in OA pathogenesis has made the chondrocyte the target of OA therapy (50), aiming to inhibit the synthesis and activity of cytokines and proteinases or enhance the repair capacity.

1.5.1 Lipid mediators/arachidonic acid

Mounting evidence point to proinflammatory cytokines IL-1β and TNF-α as key players in cartilage degradation. IL-1β and TNF-α stimulate the synthesis of proinflammatory prostaglandins through the induction of the catalyst enzyme cyclooxygenase (52). Prostaglandin E_2 has been reported to have a dual effect in cartilage, both enhancing collagen type II and proteoglycan synthesis by counteracting IL-1, and stimulating the production of degrading MMPs (53).

When a cell suffers injury or other stimuli, phospholipases in the cell membrane are de-esterified into arachidonic acid by phospholipase A2. The enzymes cyclooxygenase (COX) -1 and -2 convert the arachidonic acid to prostaglandin E_2 (PGE_2) while 5-lipoxygenase (5-LOX) along with 5-lipoxygenase activating protein (FLAP) convert arachidonic acid to leukotriene A_4 (LTA_4). Leukotriene A_4 hydrolase rapidly convert LTA_4 to LTB_4. The level of eicosanoids like PGE_2 and LTB_4 in synovial fluid is elevated in inflammatory joint disease like osteoarthritis and rheumatoid arthritis. The source is primarily the synovial membrane (54,55), but the chondrocyte can synthesise PGE_2 under IL-1 and TNF-α stimulation (50). Non-steroidal anti-inflammatory drugs (NSAIDs) are widely used in the treatment of OA and through the inhibition of COX and they lower the level of PGE_2 in the synovial

fluid. Studies have indicated that inhibiting the COX-pathway will shunt the conversion over to the 5-LOX arm resulting in higher levels of LTB_4 (53,56,57).

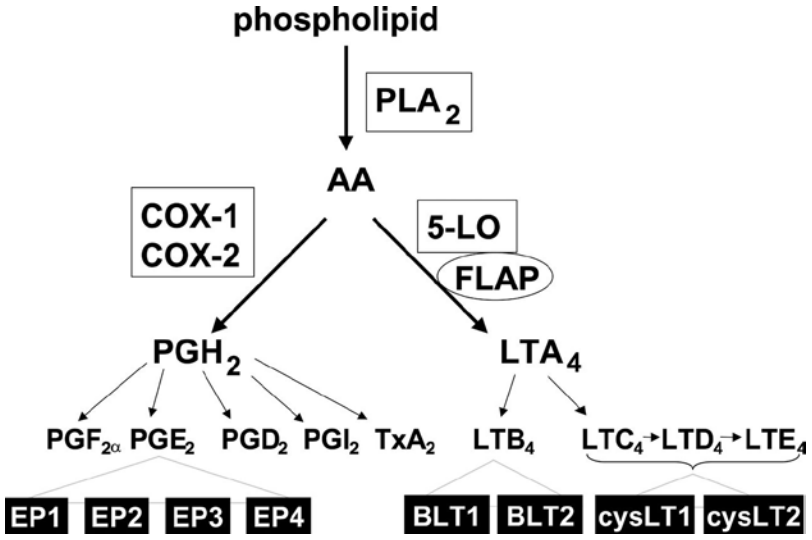

Figure 9 Arachidonic acid (AA) biosynthesis of prostaglandins (PG) and leukotrienes (LT). Black boxes indicate receptors. Figure reproduced from Charbeneau et al. 2005 (58).

The LTB_4 receptors were discovered by Yokomizo et al. who in 1997 described a G-coupled receptor that mediates chemotaxis (59). The high-affinity receptor was called BLT1 and was reported to be found primarily in leukocytes. In 2000 a low-affinity receptor was discovered and designated BLT2 (60). This receptor is ubiquitously expressed and also recognises other lipoxygenase metabolites (61). A third group of receptors has been identified in the peroxisome proliferator-activated receptors (PPARs) that reside in the nucleus and govern lipid homeostasis and inflammatory responses (62,63).

Initially, the role of the LTB_4 - BLT interaction was identified as a host defence mechanisms against infection, acting specifically in the recruitment of neutrophils. In time, it became evident that the system was also active in inflammatory joint diseases where LTB_4 upregulates the synthesis of IL-1β, TNF-α and MMPs by cells residing in the synovial membrane. Human articular chondrocytes express 5-lipoxygenase, FLAP and LTA_4 hydrolase, and are thus capable of converting arachidonic acid into leukotrienes (56,64). When chondrocytes are stimulated with growth factors such as TGF-β and 1α,25-dihydroxyvitamin D_3, the synthesis of 5-lipoxygenase, FLAP and LTB_4 increase. Naproxen, an NSAID, is reported to induce LTB_4 production that triggers an increased production of MMP1, implying the presence of a BLT1 receptor in chondrocytes (56). A potential role of LTB_4 in OA was suggested through studies on chondrocytes where blocking of the 5-lipoxygenase/FLAP pathway suppressed the levels of inflammatory mediators (65). Mice whose BLT1 gene was knocked out were protected from the development of collagen induced rheumatoid arthritis (66). The role of LTB_4 in rheumatoid arthritis was further investigated and the effect was primarily believed to be conveyed through BLT1 and BLT2 receptors on leukocytes, macrophages, dendritic cells and mast cells in the synovial membrane (67,68).

1.5.2 Vitamin D

Vitamin D is a secosteriod that occurs naturally as vitamin D_3 in mammals and as vitamin D_2 in fungi. The term vitamin is actually a misnomer as the main source is via endogenous production in sun-exposed skin where 7-dehydrocholesterol is transformed to previtamin D_3 and immediately to vitamin D_3 (cholecalciferol) via UV light and heat induction. Vitamin D is transported to the liver where it is hydroxylased to 25-hydroxyvitamin D_3, 25(OH)D_3, and this stable metabolite is measured in the serum to determine the vitamin D status. From the liver the 25(OH)D_3 is transported to the kidney where it enters cells in the proximal tubule that exhibit mitochondrial P450 1α-hydroxylase activity that catalyse the formation of the active hormone form; 1α,25-dihydroxyvitamin D_3 (1α,25(OH)$_2D_3$) (69). In addition to the endogenous production, vitamin D can also be obtained through fatty fish and fortified food items. Vitamin D tightly regulates the calcium and phosphorous homeostasis, securing the calcium level in plasma and bone mineralisation (70). It acts via the vitamin D receptor (VDR) that complexes with retinoic acid X receptor (RXR) in the nucleus (71). In the small intestine the 1α,25(OH)$_2D_3$ regulate calcium and phosphorus absorption, while in a state of dietary calcium deficiency the 1α,25(OH)$_2D_3$ will act on the bone to release precious calcium and phosphorous stores into circulation to maintain adequate physiological levels. The binding of 1α,25(OH)$_2D_3$ to VDR also regulates cellular differentiation and proliferation (72).

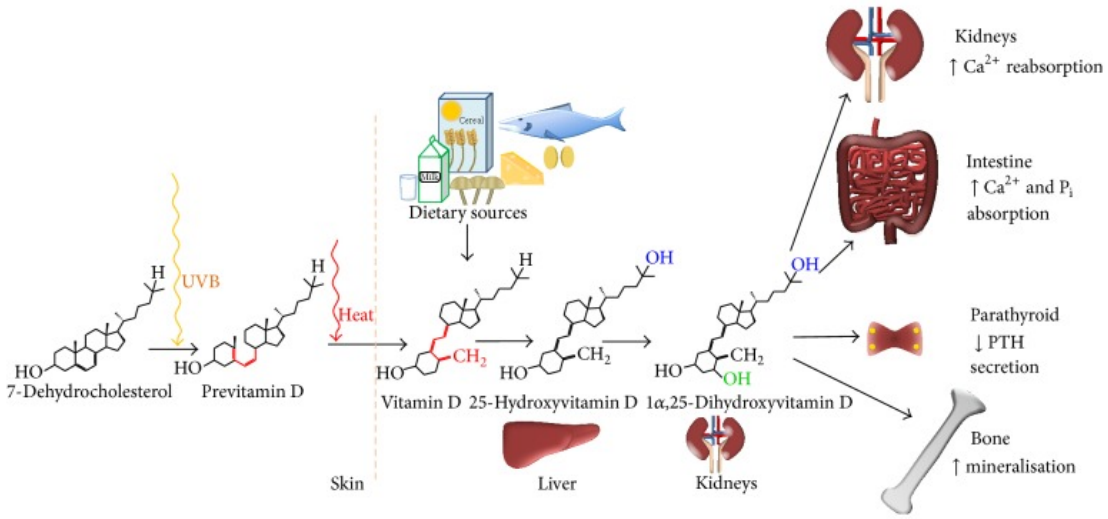

Figure 10 The biosynthesis of vitamin D and major effects on organs. Pi: inorganic phosphate, Ca: calcium, PTH: parathyroid hormone. The figure is reproduced from Mabey et al. 2015 (73).

Clinical studies have indicated that inflammatory joint diseases, like osteoarthritis and rheumatoid arthritis, are more prevalent in people deficient of vitamin D (73). At the same time, the level of 1α,25(OH)$_2D_3$ in the synovial fluid has been shown to be elevated in patients suffering from inflammatory joint conditions (74). Clinical studies have tried to establish whether vitamin D supplements reverse this effect or perhaps has a protective effect in those with symptoms of osteoarthritis. The results are so far controversial, ranging from no effects to improvement of pain and activity scores (75), and even structural effects (76). Recent studies have failed to detect any impact of vitamin D on radiographic osteoarthritis or joint space narrowing (77).

The role of vitamin D in inflammatory joint disease is likely played by local (extrarenal) production of 1α,25(OH)$_2D_3$ that acts in an auto- or paracrine matter, not influencing the pool of circulating

1α,25(OH)$_2$D$_3$ (78,79). Joint tissues and cells including osteoblasts, osteoclasts, synovial fibroblasts and macrophages exhibit 1α-hydroxylase activity (80) and the vitamin D receptor has been detected in cartilage and synovial tissues, most notably during inflammatory conditions (81,82). In the kidney the 1α-hydroxylase is regulated by its end product and activated by calcitonin and parathyroid hormone (72), while in joint tissues the 1α-hydroxylase is regulated by less elucidated mechanisms (83,84). The single gene encoding both renal and extrarenal 1α-hydroxylase, is CYP27B1 (69). When expressed in the kidney, the gene is upregulated by parathyroid hormone and downregulated by fibroblast growth factor 23 (FGF23) (85). Regulation of CYP27B1 and VDR expression has been investigated in primary human osteoblasts, where calcium had a positive effect on CYP27B1 gene expression, while VDR was dose dependently upregulated by 25(OH)D$_3$ and 1α,25(OH)$_2$D$_3$ (84,86).

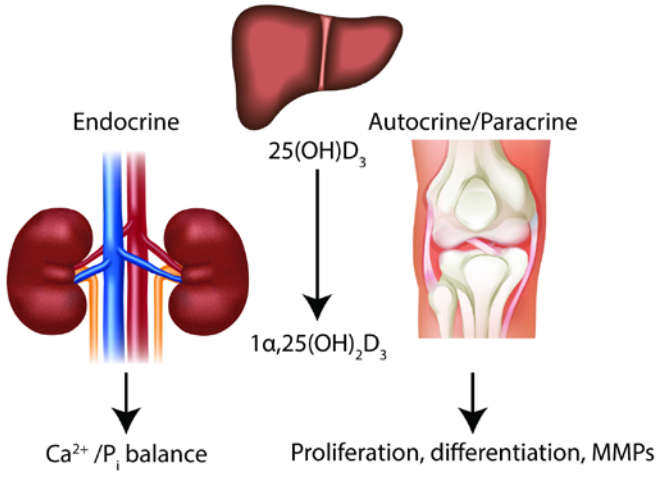

Figure 11 Endocrine vs autocrine/paracrine effects of vitamin D. Vector drawings are from Colourbox.com.

1α-hydroxylase also acts on the fungi-derived vitamin D$_2$ (ergocalciferol), which is used as a vitamin D supplement. It has been questioned whether vitamin D$_3$ and vitamin D$_2$ are equivalent as vitamin D supplements, as some argue that the long term effect of vitamin D$_3$ supplementation is more efficient than that of vitamin D$_2$ (87).

1.5.3 Cell-surface receptors

The chondrocyte express a multitude of cell-surface receptors involved in communication between the cell and its surrounding matrix. A number of such receptors have been suggested as potential biomarkers to predict chondrogenesis *in vitro* or improved clinical outcomes after cell-based therapies.

Integrins

Integrins are heterodimeric cell-surface receptors composed of one α and one β subunit. These receptors are involved in cell-cell and cell-matrix interactions, and mediate the attachment of the cell to extracellular matrix and signal transduction from extracellular matrix to the cell. In mammals, 18 α and 8 β subunits are known, while chondrocytes express a subset of 8 α and 3 β subunits (88,89). Integrins functionally interact with extracellular matrix and the cytoskeleton, as well as growth factors and their receptors (90). Typical ligands are collagen, laminin, vitronectin, osteopontin and fibronectin (91). Through their short cytoplasmic tail, integrins signal downstream by recruiting an array of signal substances including focal adhesion kinase (FAK) and mitogen-activated protein kinase (MAPK) pathways (92).

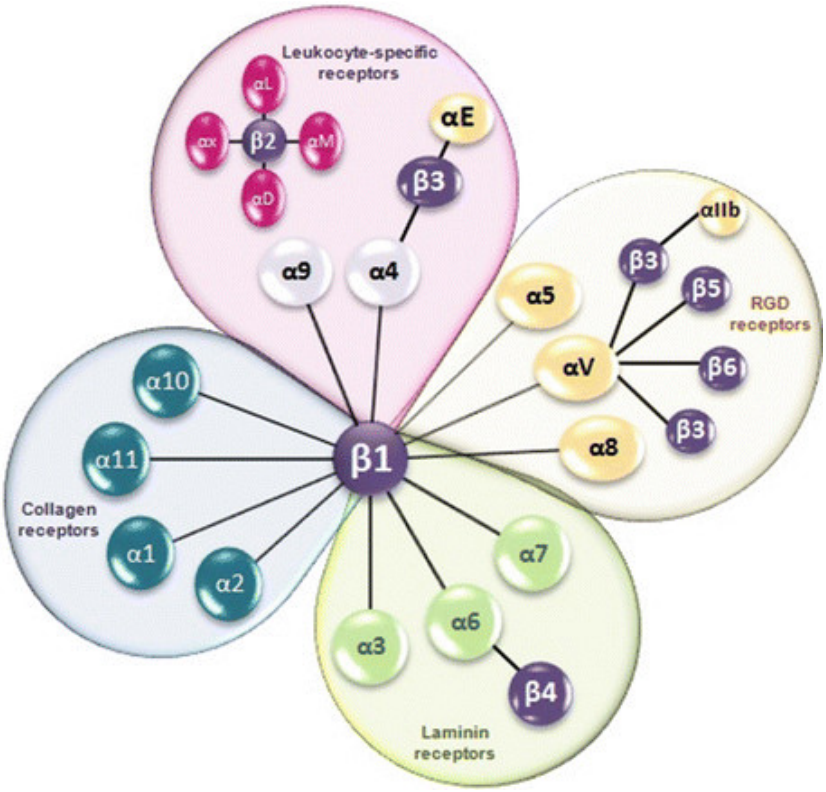

Figure 12 Integrins. Figure reproduced from Moura-Neto et al. 2014 (93).

Integrins have no intrinsic enzymatic activity but become activated after binding of the extracellular ligand and undergo conformational changes that propagate across the membrane to activate cytoplasmic kinase- and cytoskeleton signalling cascades that control cell attachment, response to mechanical load, responsiveness to growth factors, differentiation and survival (90,94). Normal chondrocytes express integrins α1β1, α2β1, α5β1, α6β1, α10β1, αVβ3 and αVβ5 while OA chondrocytes express high levels of β1 and all of the α chains (92,94).

The central role of integrins in communication between the chondrocyte and the matrix has elicited an interest in these receptors as potential key players in chondrogenesis. In cell culture studies the expression of integrin β1 and integrin α3 has been linked to increased chondrogenic potential (95–97).

Other cell-adhesion molecules and cell-contact receptors

Other relevant cell adhesion molecules more involved in cell-cell interactions comprise N-cadherin (CDH2) and neural cell adhesion molecule (N-CAM), which play vital roles during the initial cell condensation stage of chondrogenesis, where cell-cell communication is crucial. N-cadherin is a calcium-dependent glycoprotein in the cadherin superfamily that recognise cadherin molecules on other cells, thus facilitating cell condensation. Downregulation of N-cadherin is necessary to switch from condensation to differentiation. The calcium independent glycoprotein N-CAM is a member of the immunoglobulin superfamily. N-CAM recognise other cell adhesion molecules and regulates the condensation process. Both molecules are lost upon differentiation and continued expression is linked to inhibition of chondrogenesis (9).

The surface molecule CD44 is the main receptor of hyaluronic acid and crucial for the maintenance of cartilage homeostasis (98). By retention of hyaluronan/proteoglycan/link protein aggregates at the

chondrocyte surface (99), CD44 contributes to the structural organisation of the pericellular matrix (100) as illustrated in Figure 2. During inflammatory conditions, the intercellular adhesion molecule 1 (ICAM-1, also termed CD54) is induced, and functions as a hyaluronic acid receptor along with CD44 (101). In a study comparing chondrogenic potential of stem cells from various sources, including bone marrow and subcutaneous fat, the expression of ICAM-1 was a predictor of poor chondrogenic potential (102), while in another study the increased expression of ICAM-1 was linked to a favourable chondrogenic potential of umbilical cord blood stem cells compared to bone marrow stem cells (103). Higher expression of tetraspanin (CD151) and activated leukocyte cell adhesion molecule (ALCAM, CD166) by chondrocytes have been associated with increased matrix production during 3D culture (97), while sorting out CD146 (melanoma cell adhesion molecule, MCAM) positive chondrocytes was linked to improved chondrogenesis (104). Specific selection of cells with high expression of CD271 (low-affinity nerve growth factor receptor) and low expression of CD106 (vascular cell adhesion molecule, VCAM-1) has also been associated with improved quality spheroids (105).

TGF-β receptors superfamily

In chondrocytes and MSCs the TGF-βs are major regulators of chondrocyte lineage selection and cartilage matrix synthesis while actively blocking terminal differentiation and hypertrophy (14,90). Mechanical loading of cartilage triggers the release of TGF-β. In the absence of loading, this mechanism can be mimicked by the addition of exogenous TGF-β. BMP-2, -4 and -6 have the ability to enhance the chondrogenic effect of TGF-β, where BMP-2 has been shown to be most efficient at inducing chondrogenesis in the combination with TGF-β3 (106,107).

The transforming growth factor-β (TGF-β) and bone morphogenetic protein (BMP) both belong to the TGF-β superfamily of polypeptide signals that the human organism rely on to control a range of cell functions including differentiation, tissue repair and inflammation. The TGF-βs and BMPs bind a receptor complex made up of one type I receptor subunit (activing receptor-like kinase, ALK 1-7) and one type II receptor subunit. More specifically the TGF-βs utilise ALK1 or ALK5 (transforming growth factor-β receptor I, TGFBRI) as the type I receptor and TGFBRII as the type II receptor, while BMPs signals through ALK1, ALK2, ALK3 (BMPR1A) or ALK6 (BMPR1B) as the type I receptor and BMPRII as the type II receptor. The membrane-anchored type III receptor, TGFBRIII or betaglycan, captures TGF-β for presentation for the signalling receptors I and II (108,109).

The type I receptor involved in the signalling governs the downstream activation cascade. When the signals are transmitted via ALK1, ALK2, ALK3 or ALK 6, the SMAD1/5/8 complex is phosphorylated downstream, while SMAD2/3 is phosphorylated by ALK4, ALK5 and ALK7 (14).

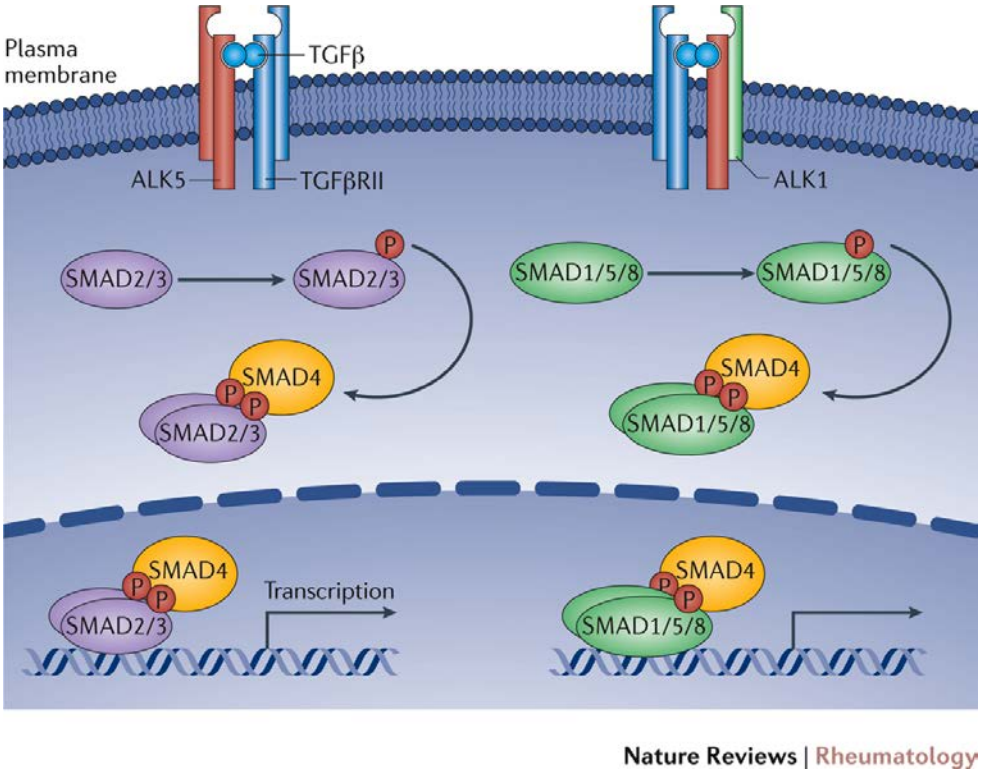

Figure 13 TGF-β signalling. Reprinted by permission from Macmillan Publishers Ltd: Nat Rev Rheumatol. (109)*, copyright (2017).*

This means that the TGF-βs can activate both the SMAD1/5/8 (via ALK1) and the SMAD2/3 pathway (via ALK5), while BMPS activate SMAD1/5/8 (14,110). The ALK5 pathway is the main pathway in healthy and young cartilage allowing TGF-β to act as a chondroprotector, while in ageing cartilage and osteoarthritis the ALK1 pathway is upregulated turning TGF-β into a pathogenic factor (109).

2 Aims of the thesis

The overall aim of the work presented in this thesis was to investigate the biology of certain inflammatory and other cell signalling receptors on human articular chondrocytes and to ascertain whether such receptors play any relevant role in chondrogenesis, cartilage homeostasis and/or cartilage repair.

The specific subaims were:

1) To explore the expression of functional leukotriene B_4 receptors in human articular chondrocytes and to elucidate if receptor engagement had effects on chondrogenesis and other major chondrocyte functions.
2) To determine whether human articular chondrocytes express the vitamin D receptor and the vitamin D activating enzyme 1α-hydroxylase, and to elucidate if vitamin D exerts relevant effects on chondrocyte functions and cartilage homeostasis.
3) To search for putative biomarkers associated with superior chondrogenic potential for patient stratification, with emphasis on integrins and other cell contact receptors.

3 Methodological considerations

This chapter aims to discuss important aspects related to the materials and methods used in this thesis work, as well as to comment on some strengths and limitations associated to the chosen methodology.

3.1 Biological material

In Papers I and II cartilage tissue harvested from patients undergoing total knee replacement, was used for experimentation. Patients were invited to donate tissue only if the medical history gave no reason to suspect rheumatoid origin of the arthritis. The cartilage was harvested from a healthy looking area of the joint, usually the lateral femoral condyle. The tissue was still regarded as OA tissue since degeneration severe enough to warrant a joint replacement is generally considered a disease of the whole joint, including the healthy looking areas. As indicated in Figure 3 of the introduction, the composition of ageing and OA cartilage is different from that of normal young cartilage. Although the incidence of knee OA is higher in women, the cartilage composition appears to be comparable between the genders (111). The results from these studies are thus limited to the context of OA cartilage and any attempt of extrapolating to normal cartilage conditions must be performed with caution.

In paper III, and some experiments in paper I, surplus chondrocytes from ACI procedures were used for experiments. These cells are generally considered healthier than the cells generated from knee replacement surgery since the indication for ACI is a localised cartilage defect and not a whole-joint disease. Overall, the surplus cells proliferated more rapidly than the cells harvested from OA knees as an indicator of younger and healthier cells (112).

The research project was evaluated by the regional ethics comity (REC). Since we have several ongoing projects in our group, we hold a permission to include chondrocytes from knee replacement procedures and ACI procedures in a biobank, given the written approval by the patient. A REC approval was obtained for the use of the cells for the research questions outlined in the different papers, serving as an external evaluation of the research question and ethical considerations. The REC evaluation ensures that the obtained samples are used for research that is meaningful for the public and the patient, and safe for the participating patients.

3.2 Cell culture systems

Freshly isolated chondrocytes (directly harvested from enzymatic digestion of the tissue) and short-term suspension cultures hold a differentiated stage, demonstrated by gene expression profiles comparable to that of native chondrocytes (113). However, cartilage cells dedifferentiate after successive cell divisions in adherent cultures, losing their original traits and switching to a more fibroblastic phenotype characterised by increased production of collagen type I and versican, and decreased amount of collagen type II and aggrecan (114). Consequently, findings using monolayer cell cultures should be cautiously interpreted, and cannot be directly extrapolated to native cartilage conditions. To circumvent the problem of cell dedifferentiation *in vitro*, we introduced a suspension culture model in Paper II enabling experimentation of cells holding a native phenotype (115).

Cells were isolated from cartilage by mincing the freshly harvested cartilage into sub ~1mm^3 pieces under sterile conditions followed by enzymatic digestion in collagenase XI at a final concentration of 1.25 g/L. The cartilage was approximately 95 % degraded after 4-6 hours and the cells were separated

from the collagenase and undigested pieces by a 70 µm cell strainer and subsequent centrifugation before they were resuspended in fresh medium. The freshly isolated cells were mixed with Trypan blue solution, and the number of live and dead cells was assessed using a haemocytometer. Initial experiments using overnight digestion resulted in up to 50 % dead cells. The experimental conditions were optimised and at 4-6 hours digestion, the amount of dead cells was negligible.

Henceforth the cells were used in static suspension cultures or expanded in monolayer cultures. In static suspension cultures, 4×10^5 freshly isolated cells were propagated in 800 µL medium in ultra-low attachment 24-well plates. After 24 hours of equilibration, the cells were stimulated and harvested. This approach allowed experiments on differentiated chondrocytes. However, a limitation of the static suspension culture is the relative low amount of cells harvested from each cartilage specimen. In order to draw samples for both protein- and gene assays, a minimum of 4×10^5 cells per experimental condition was necessary. Generally, this required cartilage samples exceeding 1-2 grams. Another drawback is the relative high amount of cartilage-derived extracellular matrix proteins in the cultures, which could represent a problem when analysing results of Western blots.

The 3D culturing method supports redifferentiation of chondrocytes back to a chondrocyte-like phenotype (9). Our lab employ two different methods to prepare 3D cultures: the pellet culture system and the hanging drop system (116). In the hanging drop method, drops of 2×10^4 chondrocytes are applied to the lid of a 24-well plate, next the lid is inverted so that the drops are suspended from the lid driving the chondrocytes to converge in the tip of the drop by gravitational forces. To avoid dehydration the hanging drop pellets are suspended over wells filled with medium. The pellet method applies conical-bottom 96-well plates that are coated with poly-HEMA to prevent cell adherence. After dispensing 5×10^4 chondrocytes/150 µL medium/well, the plate is centrifuged at 1100 × g for 10 minutes to allow the formation of pellets. Alternatively, an ultra-low attachment 96-well round-bottom plates is applied, followed by 5 min centrifugation at 500 × g. In either hanging drop or pellet approach, early formed spheroids are transferred to low attachment 24-well plates in chondrogenic medium after two days in order to secure sufficient access to nutrients. For both methods the spheroids are incubated in low (2.5 %) O_2 conditions further promoting chondrogenic differentiation (117).

Previous studies have shown that if the 3D differentiation is performed during the early expansion phase (within 10 days of initial plating), no growth factors are necessary to generate cartilage-like micro-tissues. In our setup we generally needed to expand the chondrocytes for 21 days or more to obtain sufficient amount of chondrocytes for the planned assays, and in this setting growth factors were necessary to induce chondrogenesis (118). This is evident in paper I where ACI surplus cells were used for spheroid formation in the absence of dexamethasone and TGF-β. The omission of growth factors was based on the aim to find effects of LTB_4 alone, but the result was that the spheroids formed very little matrix in the absence of growth factors. In Paper II, the spheroids were prepared from OA chondrocytes and propagated in serum-free medium containing TGF-β3, BMP2 and dexamethasone. In addition $25(OH)D_3$ or $1\alpha,25(OH)_2D_3$ was added to evaluate effect on chondrogenesis. The untreated spheroids show some ability to produce matrix, but far less than the spheroids prepared from ACI surplus chondrocytes in Paper III. These findings reflect previous reports stating that OA chondrocytes have lower capability of recovering normal tissue phenotype compared to healthy chondrocytes (119).

3.3 Immunolabelling

3.3.1 Immunohistochemistry

In Papers I and II, immunohistochemistry was applied to evaluate the expression and distribution of proteins of interest in cartilage and spheroid sections. Briefly, the method includes formalin fixation of the tissue, sectioning and mounting onto slides, blocking of background signalling, peroxidase quenching, incubation with a specific primary antibody, incubation with horseradish peroxidase (HRP) polymer conjugated secondary antibody and finally development by DAB chromogen. For the BLT1 and BLT2 receptors investigated in Paper I, the staining was apparent by this protocol alone. However, this was not the case for the vitamin D receptor interrogated in Paper II. The formalin fixation of the tissue sections can induce modification of the antigens and loss of the ability of the antibody to react with the antigen. The recommended method to restore the antigen is by heat-induced antigen retrieval (120). Unfortunately, due to the nature of the cartilage tissue, the heat makes the sections detach from the glass. We tested an alternative approach using antigen retrieval at lower temperature. When the temperature is decreased the time must be increased to obtain sufficient antigen retrieval (121) and the sections were incubated overnight in 60° C retrieval buffer (122). Using this more gentle protocol, we still experienced detachment of the sections. We tested both Tris-EDTA (pH 9.0) and citrate (pH 6.0) retrieval buffers with similar outcomes, that is; no staining of VDR, while 1α-hydroxylase was apparent in the superficial layers of OA cartilage sections. The positive control tissue (intestinal mucosa) was stained by both VDR and 1α-hydroxylase. We continued troubleshooting the VDR staining by testing an antigen retrieval protocol suggested by Tetlow et al., where sections were incubated in 2 M HCl at 37° C for 30 minutes (123), but in our hands this did not result in staining of the VDR. Lastly we attempted antigen retrieval by enzyme digestion using hyaluronidase and pronase as previously described (124), but still we failed to obtain any staining of the VDR in OA cartilage and spheroids. The VDR antibody was selected based on the comprehensive paper by Wang et al. (125), where the several VDR antibodies were compared in terms of sensitivity and specificity. The rigorous validation described in this paper is not available for all antibodies, and the experimental setup can be extensive since particularly a true negative control sample, usually from knockdown animals, can be challenging to obtain. An alternative approach of validation is to immunoblot for bands of the correct molecular weight (126), and this method was applied to the antibodies used in Paper II. True positive controls are more easily obtained, exemplified by the intestinal mucosa used in Paper II where the expression of both 1α-hydroxylase and VDR is well established. Since the sections of intestinal mucosa were stained through all experimental conditions, while the cartilage sections remained unstained, we concluded that the VDR is either absent or expressed below the detection threshold of the assay. In all experiments, sections devoid of the primary antibody were used as controls of unspecific staining of the secondary antibody.

3.3.2 Immunocytochemistry

Immunolabelling by specific antibodies in cultured cells was used in Papers I and II. This method enables visualisation of the protein within the cell and as demonstrated in Paper II, the subcellular distribution between cytoplasm and nucleus can be evaluated. Cells were expanded on glass slides until confluent, fixed and permeabilised by brief incubation in ice-cold methanol followed by blocking of background signals using a 2 % human serum albumin solution. Next, the slides were successively incubated with primary antibody and a fluorophore-conjugated secondary antibody followed by image acquisition via a fluorescence microscope. The approach for validating immunocytochemistry is

equivalent to the outline in section 3.3.1, and the immunoblotting serving as validation for immunohistochemistry is thus valid for the immunocytochemistry. Sections lacking of the primary antibody were used as negative controls of the secondary antibody.

The VDR resides in the nucleus in the absence of $1\alpha,25(OH)_2D_3$, but to a greater extent in the presence of the ligand (127,128). In previous studies this has been demonstrated by Western blot of nuclear vs. cytoplasmic fractions of cells (129), but the effect has also been visualised by immunocytochemistry in human skin fibroblasts and rat kidney cells (125,130). In order to examine the activity of the VDR in chondrocytes we expanded chondrocytes on glass slides before treatment with $1\alpha,25(OH)_2D_3$ (10^{-7} M) for 2 hours before permeabilisation and fixation of the chondrocytes. This assay successfully demonstrated the nuclear translocation of the vitamin D receptor.

3.3.3 Western blot

Western blot (immunoblotting) was used to evaluate receptor engagement in Paper I by probing for phosphorylated protein kinases downstream of the receptor. In Paper II, immunoblotting was used to validate that the antibodies used for immunohistochemistry produced bands of the correct molecular weight, and for semi-quantitatively assessment of receptor and enzyme regulation. The protocol was based on the principles thoroughly outlined by Bass et al. (131). Protein samples were prepared using the RIPA (Paper I) or NP-40 (Paper II) lysis buffer to obtain whole cell extracts. A ratio of 1 mL lysis buffer per 0.5 - 1 × 10^7 cells proved vital to obtain sufficient protein concentrations. As we were probing for cell-associated proteins, it was necessary to remove the extracellular matrix prior to cell lysis. For native cartilage, this was done by enzymatic digestion of the tissue and subsequent harvest by centrifugation. However, we experienced that the levels of extracellular matrix proteins were overriding in samples even after suspension subcultures. This can be observed in Figures 1 and 2 in paper II where the loading control β-actin (a cell-associated protein) is substantially lower expressed in suspension culture samples compared to monolayer samples, despite that the amount of input protein being compensatory increased in the former. This issue has previously been described in RNA extraction protocols (132). Others have noted that protein input must be increased in order to detect VDR in tissue compared to cells, and that a hyperosmolar lysis buffer facilitating release of DNA-bound protein may be important (133). The suspension cultures in Paper II were homogenised by centrifuging samples through a QIAshredder column, but this did not reduce the difference in β-actin expression. The difference in expression of the loading control between the different culture conditions implies that a direct comparison of these samples is inadequate. An alternative normalisation method, that may have produced more consistent results in our setting, is the use of Coomassie staining (134). A protease inhibitor was used in all conditions, and when probing for phosphoproteins (Paper I) a phosphatase inhibitor was included as well. Lysates from osteoblasts were used as positive controls of antibody specificity in Paper II.

Equal amounts of protein from each sample type were separated by gel electrophoresis and the proteins blotted onto PVDF membranes. The PVDF membrane has high affinity for proteins, including the antibody-proteins. Thus, after blotting, the remaining surface was blocked using milk proteins to prevent nonspecific binding of antibodies during subsequent steps. Finally, membranes were incubated with primary and secondary antibody followed by chemiluminescence detection using a charge-coupled device (CCD) camera. During initial runs of the protocol for Paper II, some unexpected bands of low molecular weight appeared on the final images, indicating digestion of the

sample. This feature disappeared upon the use of a fresh batch of protease inhibitor, producing the images presented in Paper II. Of note, many proteins exist as isoforms and depending on the primary antibody it may or may not be specific for one or more of the isoforms (135). In the case of the latter, multiple bands are expected as specified by the supplier or in the literature.

3.4 Polymerase chain reaction

The polymerase chain reaction (PCR) exploit the **DNA polymerase** enzyme that makes copies of DNA. By repeating the copy process multiple times, low amounts of DNA or cDNA are amplified to levels that can be detected using gel electrophoresis (RT-qPCR) or fluorescence based detection methods (qPCR).

3.4.1 RNA extraction

Cellular processes like growth, differentiation and survival can be assessed by measuring the amount of messenger RNA in a sample (136). Attempts were made to extract total RNA from cartilage and spheroids by crushing the tissues in a mortar under liquid nitrogen, followed by extraction using phenol-based lysis buffers. This method was flawed by low reproducibility, low RNA yield and phenol contamination as judged by Nano-Drop spectrophotometry evaluation and qPCR validation experiments. An optimised protocol including enzymatic digestion of cartilage, harvest by centrifugation and immediate disruption and homogenisation in a phenol free lysis buffer, resulted in increased RNA yield increased and low contamination (113). Freshly isolated chondrocytes or intact spheroids were processed by mixing the sample and lysis buffer in a 2 mL Eppendorf tubes containing a 5 mm stainless steel ball, and subsequent disruption of samples in a TissueLyser bead mill for 2.5 min at 25 Hz. To homogenise and remove insolubles the samples were passed through QIAshredder columns (137). In order to obtain sufficient RNA concentrations, MinElute columns were used for subsequent purification of freshly isolated chondrocytes, suspension cultures and spheroids. Extraction of RNA from chondrocytes expanded in monolayer cultures is straightforward as there is less interference from extracellular matrix (132), the cells can be lysed directly in the culture vessel and RNA extracted without further disruption or homogenisation steps to yield high concentrations of clean RNA. On-column DNase digestion was performed on all samples to eliminate contaminating DNA.

3.4.2 Reverse transcription to cDNA

The DNA polymerase acts on double stranded structures and since RNA is single stranded the **reverse transcriptase** enzyme is used to create double stranded cDNA. The reverse transcription (RT) process should reflect the amount of RNA input, but the presence of inhibitors can influence the efficiency of the reverse transcriptase. Validation of the RT was performed using a dynamic range test where RNA was diluted in a five step 1:3 dilution series. Each dilution was transcribed to cDNA and amplified by qPCR using a probe with high gene expression level (the reference gene) and a probe with low gene expression level (138). The results indicated that the undiluted samples were not amplifying at a linear range and the RNA was diluted 1:3 for all subsequent RTs.

3.4.3 Reverse Transcriptase-PCR

In reverse transcriptase PCR (RT-PCR), the cDNA is amplified in the presence of oligonucleotides (primers) designed to specifically bind the gene of interest (GOI). The primer serves as a starting point for the DNA polymerase, enabling amplification of the GOI. After 35 cycles of amplification, the

end-product is separated by gel electrophoresis and if the primers have been correctly designed, a band matching the size of the expected product can be visualised. A reference gene is amplified to control loading and purity, as it will produce an extra band in the case of contaminating DNA. The end-product corresponds to the plateau phase of the reaction where the substrates are limited or exhausted. For various reasons this plateau phase can differ between identical samples and this limits the use of RT-PCR for quantification purposes. It is however useful for determining the presence of transcribed genes as we did in Papers I and II (139).

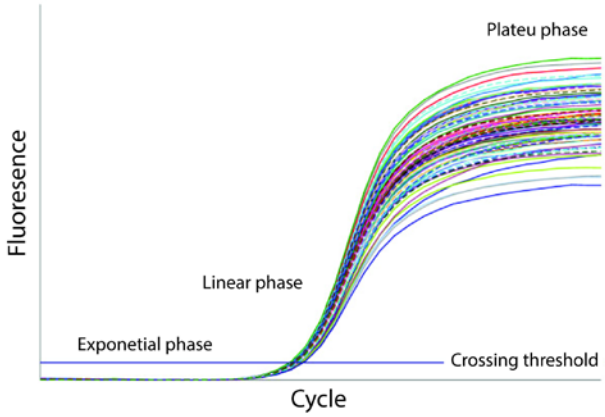

Figure 14 End stage PCR. Illustration of how identical samples can have variable plateau phases. Figure adapted from Larionov et al. 2005 (140).

The primers used in Paper I had been validated in previous publications (141), while for Paper II it was necessary to design primers that would identify all homologues of the vitamin D receptor. Messenger RNA sequences that were common in all homologues were identified using BLAST (142) and tested in the Primer3Plus online software (143). Two different primer pairs for VDR and 1α-hydroxylase were tested and the pairs producing the clearest band of the correct size were used in the final setup.

3.4.4 Quantitative real-time qPCR

Quantitative real-time qPCR (qPCR) depends on the same basic ingredients as the RT-PCR reaction: DNA polymerase, primers and a DNA or cDNA template. In addition, the qPCR reaction incorporates a dye that emits fluorescence proportional to the amplification. The fluorescence is detected by the qPCR platform and the amplification reaction can thus be followed in real-time. The qPCR reactions in this study are based on hydrolysis probes. The hydrolysis probe is an oligonucleotide designed to bind the template downstream of one of the primers and is equipped with a fluorescent dye and a quencher molecule. The quencher molecule prevents the dye from emitting fluorescence as long as they are proximate. During amplification, the polymerase cleave the probe, separating the quencher from the dye allowing for emission of fluorescence.

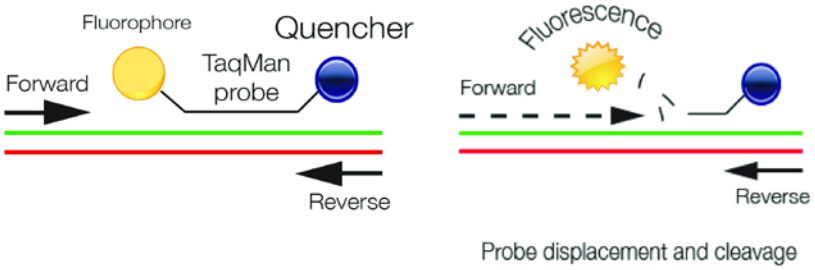

Figure 15 Hydrolysis (TaqMan) probe. Illustration of how the quencher is release during amplification, thus allowing the reporter to emit fluorescent light that is captured by the qPCR platform. Figure adapted from cogentech.it (144).

As long as templates (cDNA) and reagents are unlimited, the templates are doubled for each cycle and fluorescence thus increase exponentially. When reagents are restricted, the fluorescence follow a linear curve and once depleted the reaction enters the plateau phase.

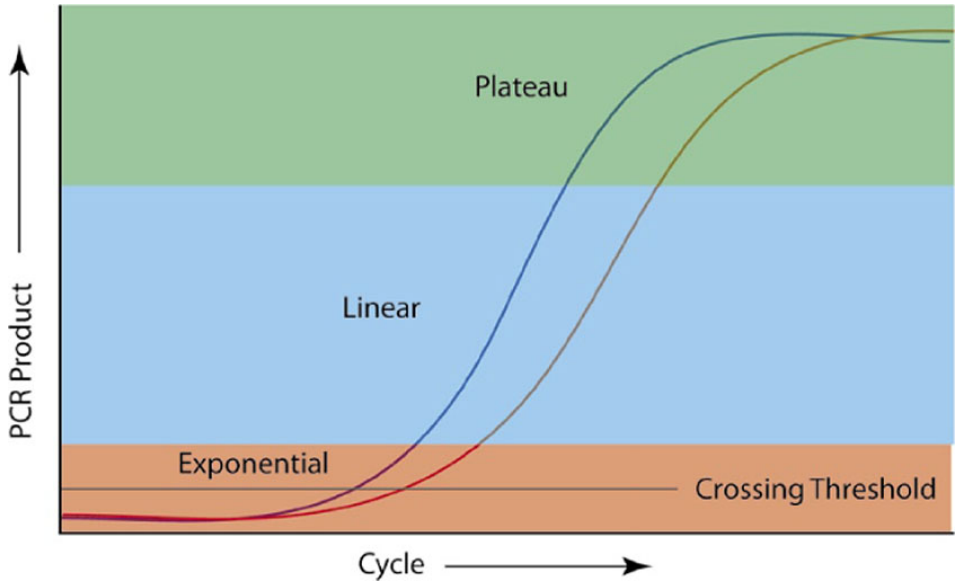

Figure 16 qPCR phases. The figure is reproduced from VanGuilder et al. 2008 (139).

The hydrolysis probes used in this study are pre-optimised from the vendor and further validation of efficiency is reported to be redundant (145), particularly because the validation process itself has some pitfalls. Nevertheless, a five step 1:10 dilution series of cDNA from representative samples was prepared and each dilution was subjected to qPCR using probes for high (reference gene) and low (COL2A1) expressing genes. Excluding the undiluted sample resulted in dilution curves of efficiency within the recommended 90 to 110 %.

Since RNA was extracted from various matrices using slightly different methods it was important to identify one or more references genes that were expressed uniformly across the samples (146). Validation of the optimal reference gene was performed by using samples from all interrogated matrices (freshly isolated chondrocytes, suspension culture chondrocytes, monolayer chondrocytes and spheroids) and subjecting them to qPCR using an array of potential reference genes. The results

were analysed using the NormFinder and geNorm tools. GAPDH proved to be a stable reference gene in studies of monolayer samples, while in studies including cartilage, suspension culture and spheroid samples the RPL13A proved to be more stably expressed. Combining two or more reference genes did not increase the stability.

Plates were loaded using the sample maximization method (147), eliminating the need for interplate calibrator samples. The Cq values of the gene of interest were normalised by subtracting the Cq value of the reference gene ($\Delta Cq = Cq_{reference} - Cq_{GOI}$). This resulted in ΔCq values that reflect the regulation, i.e. higher values mean higher expression and vice versa. The ΔCq values were used for statistical testing and were plotted directly using bee swarm plots. Significant up- or downregulation of genes was evaluating using t-tests in setups of few samples and genes (Paper I). For larger assays (Paper II and III), an ANOVA was applied, followed by Dunnett's post hoc test where multiple experimental conditions were compared to one control condition (148).

It is common to present the data as fold change of expression compared to a control sample, the fold change can be calculated using the $2^{\Delta\Delta Cq}$ formula where ΔΔCq = mean(ΔCq treated) – mean(ΔCq control). The drawback of representing fold change is that any upregulation will be visualised nicely taking a value of 1 or more, while downregulation will have a value restricted between 1 and 0. If the axis representing the fold change is set to log scale, the up- and downregulation will be given equal weight, this option was used in Paper I. Another option is to log the fold-change; this will also give equal weight to the up- and downregulation. However, if a log base 2 is used for the log transformation, the result is the ΔCq value. Using fold change and log base 2 is thus mathematically redundant. Displaying the ΔCq or ΔΔCq values directly will correctly reflect the regulation and this option was used in Paper II and Paper III. To ease the interpretation of the results the results in paper III were shifted along the scale so that the control sample centers on zero for each gene. Relative quantification justifies the comparison of different samples analysed with the same probes, but does not allow for comparison between different probes (149).

In paper II, we compared samples from different matrices from the same donors (treated and untreated suspension culture chondrocytes and monolayer chondrocytes). Donor to donor variation exceeding the difference in gene expression is a common challenge, which we encountered in Paper II. A solution was suggested by Willems et al. (150) who used mean centring and autoscaling to minimise the effect of donor-to-donor variation and focus on the difference in gene expression. The authors propose to apply the autoscaling and mean centring on log fold-change values, but this is also mathematically redundant and the same result is easily achieved by using ΔCq values directly as demonstrated in Paper II Figure S1.

3.5 Chondrogenesis

An *in vitro* chondrogenesis assay was applied in all three papers. In Papers I and II, the assay was used to evaluate the influence of LTB_4 and vitamin D respectively, while in Paper III it was used to ascertain the chondrogenic potential of donor-specific chondrocytes from ACI cell preparations.

The assay is based on the formation and cultivation of cell aggregates (spheroids) as explained in section 3.2, followed by Alcian blue staining of spheroid sections and semi-quantitative assessment of the tissue characteristics by the Bern score (151,152). The Bern score is a visual histological grading

and that correlates both with computerised histomorphometry and with GAG/DNA content. The method takes into consideration intensity and uniformity of GAG staining, cell density/matrix proportion and cell morphology. The Bern score provides a comprehensive evaluation compared to the GAG/DNA content or the simpler O'Driscoll score (153,154). Originally, the Bern score was developed based on Safranin O and Fast Green staining, but since the Alcian blue stianing generally gives more consistent results in our lab, and both stains target GAGs and proteoglycans, we have used Alcian blue staining in our studies.

SCORING CATEGORIES	SCORE
A. Uniformity and darkness* of Safranin O – Fast Green stain	
No Stain	**0**
Weak staining of poorly formed matrix	**1**
Moderately even staining	**2**
Even dark stain	**3**
B. Distance between cells / amount of matrix produced	
High cell densities with no matrix in between	**0**
High cell densities with little matrix in between	**1**
Moderate cell density with little matrix	**2**
Low cell density with moderate distance between cells and an extensive matrix	**3**
C. Cell morphologies represented	
Condensed/necrotic/pycnotic bodies	**0**
Spindle/fibrous	**1**
Mixed spindle/fibrous with rounded chondrogenic morphology	**2**
Majority rounded/chondrogenic	**3**

**Sections 3-4 µm thick*

Figure 17 Bern score. Reproduced with permission from Mary Ann Liebert, Inc., publishers: Tissue Engineering (152) *, copyright 2006.*

3.6 Cell proliferation assay

The xCELLigence system, based on impedance based growth measurement, provides a label-free real-time measurement of proliferation in adherent cell cultures. The assay was used in Paper II to assess the influence of vitamin D on chondrocyte proliferation. Equal amount of cells are seeded in the wells of a microtiter plate holding electrodes at the bottom. As the cells proliferate the impedance in the electrodes increase, enabling real-time monitoring of cell growth represented by the cell index (CI). The cell index has been compared to objective measures of cell confluence and found to faithfully represent proliferation (155). The cells are left to adhere and equilibrate for 24 hours before any treatment is added. During this time, the cells can hold slightly different growth rates, which is accounted for by normalising the cell index to the time of treatment, resulting in a *normalised cell index*. It was further shown that the base 2 logarithm of the normalised cell index corresponds to the growth rate and represents a more robust measurement (155).

4 Summary of results

4.1 Paper I

The leukotriene B_4 receptors BLT1 and BLT2 were demonstrated in human articular cartilage and in cultured chondrocytes by immunolabelling, including the subcellular localisation to the cell membrane visualised by immunoelectron microscopy. In cultured chondrocytes, expression was supported by RT-PCR using specific probes for the BLT1 and BLT2 receptors. The activity of receptor was investigated by challenging the cultured chondrocytes with LTB_4 at different concentrations (data not shown) and times, and a concentration of 10^{-8} M was sufficient to elicit phosphorylation of the downstream phosphor-p44/42 mitogen-activated protein kinase (MAPK or Erk 1/2) as detected by Western blotting. The density of the bands increased in a time-dependent matter and the addition of a specific blocker of the BLT1 receptor returned the band density to the background level. Addition of a specific blocker of BLT2 did not reduce the band density, while the simultaneous blocking of both receptors elicited the same reduction in density as blocking BLT1 alone.

We investigated the effect of LTB_4 on growth factors, MMPs and inflammatory cytokines by multiplex analysis of supernatants from cultured chondrocytes treated with 10^{-12} - 10^{-8} M LTB_4, and found that LTB_4 did not alter the secretion compared to untreated cells. Potential effect of LTB_4 on chondrocyte phenotype was evaluated by qPCR using probes targeting the chondrocyte signature genes: collagen type 1A1, collagen type 2A1, aggrecan and SOX9. The signature-gene expression profile was not affected by treating the chondrocytes with LTB_4. Next, we compared the relative expression of the BLT1 and BLT2 receptors in chondrocytes from OA samples and ACI samples and found that the expression level of both BLT1 and BLT2 was significantly reduced in the OA samples. When comparing gene expression of receptors in cartilage and cultured cells (both from OA) the expression of BLT1 was significantly reduced in the cultured cells compared to the native tissue, while the expression of BLT2 was comparable between the two groups.

Finally, we cultured chondrocytes in spheroids to test the effect of LTB_4 on redifferentiated chondrocytes and cartilage-forming capability. Sections prepared from spheroids cultured in the presence and absence of LTB_4 at 10^{-8} M for 7 days were labelled with collagen type II antibody or stained with Alcian blue to detect glycosaminoglycans, but we found no difference between the two groups.

4.2 Paper II

The biology of vitamin D in the context of articular cartilage tissue remains uncertain. In order to evaluate potential effects of vitamin D on cartilage we explored the presence of the vitamin D receptor (VDR) in cartilage (differentiated chondrocytes), cultured chondrocytes (dedifferentiated) and redifferentiated 3D cultured chondrocytes. Using immunolabelling, we failed to detect the VDR in cartilage and redifferentiated chondrocytes, while the receptor was readily detected in cultured expanded chondrocytes. Further investigation of spheroids revealed that the expression of VDR was lost shortly after initiation of 3D cultures (48 h). Western blots showed very weak bands in differentiated suspension culture samples, while expression in monolayer samples was more evident. Relevant positive controls were included in both immunolabelling and Western blotting to verify antibody specificity. In contrast, the VDR mRNA was detected in all differential stages.

The 1α-hydroxylase enzyme that facilitates the hydroxylation of 25(OH)D to the active hormone 1α,25$(OH)_2$D, was detected in cartilage (superficial layers), cultured chondrocytes and spheroids using immunolabelling. The finding was supported by Western blots, reproducing bands of the expected weight in both cartilage and cultured chondrocyte samples. PCR using primers targeting the enzyme, produced bands at the expected length in all differential stages.

To verify functionality of the VDR, cultured chondrocytes were challenged with 1α,25$(OH)_2D_3$ at 10^{-7} M or vehicle for 2 hours and labelled with VDR antibody. The treatment with 1α,25$(OH)_2D_3$, induced a translocation of the receptor from the cytoplasm to the nucleus. The activity of 1α-hydroxylase was demonstrated by challenging chondrocytes with increasing doses of the pro-hormone 25$(OH)D_3$, which resulted in dose-dependent increase in 1α,25$(OH)_2D_3$ as measured in supernatants after 24 hours.

To investigate the regulation of VDR and 1α-hydroxylase during inflammatory conditions, suspension cultures and monolayer cultures were treated with IL1β (10 ng/mL) or 1α,25$(OH)_2D_3$ (10^{-8} M) for 24 hours and protein levels were assessed by Western blots. Neither IL1β nor 1α,25$(OH)_2D_3$ influenced the level of VDR or 1α-hydroxylase in suspension cultures. Likewise, the expression of 1α-hydroxylase remained unchanged by both IL1β and 1α,25$(OH)_2D_3$ in monolayers. The VDR was however, significantly upregulated by IL1β, while the ligand had no such effect.

Due to the low expression of the VDR in cartilage and suspension cultures, functional assays to explore effects of vitamin D in chondrocytes were restricted to monolayer cultures where the VDR is more stably expressed. Both 25$(OH)D_3$ and 1α,25$(OH)_2D_3$ increased the proliferation in the three donors assayed. Similarly, both 25$(OH)D_3$ and 1α,25$(OH)_2D_3$ resulted in lower expression of ACAN and higher expression of VCAN. Also in the chondrogenesis assay both 25$(OH)D_3$ and 1α,25$(OH)_2D_3$ supressed matrix production in spheroids, but only when chondrocytes were expanded in the presence of the pro-hormone or active hormone for one week before establishing the 3D cultures. The equivalent effect of 25$(OH)D_3$ and 1α,25$(OH)_2D_3$ in proliferation, gene expression and chondrogenesis studies further support the action of 1α-hydroxylase in chondrocytes. The active hormone had no effect on spheroids established from chondrocytes expanded in standard growth medium, supporting that the VDR is rapidly repressed in 3D cultures.

4.3 Paper III

Surplus chondrocytes from 17 ACI procedures were examined for their intrinsic chondrogenic potential in scaffold-free 3D cultures. Chondrocytes from donors showed remarkably different abilities to form spheroids. The Bern score was used to quantitatively evaluate Alcian blue stained sections from the 17 cases followed by allocation of donors into three different groups according to the total score. The cell passage at the time of spheroid formation was evenly distributed between the groups, as were the age and gender of the patients.

Surface receptors previously proposed as markers for chondrogenesis were analysed by flow cytometry. Selected surface markers comprise CD44 (hyaluronan receptor), CD106 (vascular cell adhesion molecule, VCAM-1), CD146 (melanoma cell adhesion molecule, MCAM), CD166 (activated leukocyte cell adhesion molecule, ALCAM) and CD271 (low-affinity nerve growth factor receptor). The expression of CD44 and CD166 was uniform across groups and donors, while CD106

and CD146 displayed donor-specific patterns regardless of group affiliation. CD271 was scarcely detected and only in at a low level in groups B (intermediate group) and C (worst group). None of the markers could be used to predict the chondrogenic capacity.

We conducted a quantitative gene expression analysis using cells with the same passage as for the chondrogenesis assay. Interrogated genes included integrins α1, α2, α3, α5, α6, α10, αV, β1, β3, β4 and β5; cell adhesion molecules CD44, CDH2 (N-cadherin), ICAM1 (intercellular adhesion molecule 1), MATN3 (matrillin-3) and NCAM1 (neural cell adhesion molecule 1); and growth factor receptors TGFBR1, TGFBR2, TGFBR3, BMPR1A, BMPR1B and BMPR2. Comparing gene expression levels of group A to the expression in groups B and C revealed no significant regulation, except for TGFBR3 that was significantly upregulated in group C samples compared to group A (best group).

Using tandem-mass-tag (TMT) proteomic technology, the global spectra of cell-associated proteins was compared between three donors from group A and three donors from group C. By mass spectrometry, 2572 proteins were identified in samples from which 82 corresponded to cell-surface receptors. Semi-quantitative comparative analyses between the best and the worst chondrogenic groups revealed no significant differences in the expression of cell adhesion molecules of surface receptors, thus supporting the gene expression results. By setting the significance level at 0.1, a panel of eleven proteins were identified as differentially expressed between groups. Among the differentially expressed proteins, we found the subunits of the enzyme **prolyl 4-hydroxylase**, an enzyme that is critical in formation of 4-hydroxyproline, a vital player in the triple helix formation of collagens.

5 General discussion

Osteoarthritis is a major cause of disability and although many mechanisms of the disease have been unveiled, there is still no effective treatment or cure in sight (23,75,156,157). Age, sex, joint injury, obesity and genetic predisposition are all considered risk factors for OA (158), and ultimately the disease represents the final common pathway of age-related degenerative changes and traumatic injuries of synovial joints (23). A major issue is that the onset of idiopathic OA is insidious, and at the time of clinical presentation, the progress is too far ahead to reverse. This aspect relays specific demands to any treatment instituted as a preventive measure; the treatment must be safe and hold few or no side effects. Advanced cases of OA are successfully treated with joint replacement procedure, and a hip replacement can be performed even in young patients resulting in favourable long-term outcomes. Due to the multidirectional movement, a replacement knee joint has a limited life span and the best results are obtained in patients over the age of 65 (159). This leaves a treatment gap for the younger patients that is only partially covered by osteotomies and cell-based procedures. With the increasing incidence of OA, new treatments are urgently required as the current pharmacological options including NSAIDs, analgesics and injections have no effect on the disease progression and at best serve as a temporary suspension of the pain aspect of the disease.

The cell-based therapies introduced in the late 80's, aimed to provide a new option for patients with localised cartilage defects (39,43). As of today, the array of treatments span from microfracture, via autologous chondrocyte implantation to implantation of chondrocytes or stem cells seeded in scaffolds or scaffold-free constructs (47), all aiming to produce durable hyaline cartilage. However, no method has been proven superior (160,161) and the repair tissue remains interspaced by fibrocartilage. Clinical studies point to a common challenge; some patients respond worse to the treatment than others and being able to recognise factors associated with an inferior outcome could guide the quest for a more optimal treatment for these patients. Studies of patient characteristics largely agree on age, female gender and previous surgery as negative predictive factors (162,163). While some attempts have been made to identify biomarkers useful for stratification, few reliable or accessible markers have been identified (97,164–166).

Overall, the progression of the field is largely based on trial-error approaches and no significant advancements has been made during the last decade. Both in the case of OA and in the case of localised cartilage damage, it is of paramount importance to expand the understanding of the biological principles governing tissue homeostasis in order to acquire durable treatments. In this thesis, we have tried to gather new knowledge on the biology of cell signalling receptors that have been little studied in the context of cartilage and OA. Furthermore, we have explored the link between cell-surface receptors expression and chondrogenesis as a potential tool to select patients more likely to obtain a favourable outcome of cell-based therapies.

5.1 Paper I

NSAIDs are commonly prescribed to OA patients for pain management. Side effects in the gastrointestinal tract are well known, but the COX inhibition also leads to shunting from the prostaglandin-axis to the leukotriene-axis (167). Studies have suggested that the leukotrienes are important in modulating inflammatory joint diseases as the deletion of leukotriene-receptors halts the development of inflammatory joint disease in animals (67). Further evidence of the involvement of

LTB_4 in inflammatory joint disease include elevated levels of LTB_4 in the joint fluid (168) and the expression of the high- and low affinity receptors (BLT1 and BLT2) in synovial tissue of OA and RA patients (169). LTB_4 has also been shown to regulate the synthesis of proinflammatory cytokines and MMPs in OA synovial membrane and chondrocytes (56).

Although the main recognised function of LTB_4 is recruitment and activation of leukocytes (170), which may be the main mechanism of the protective effect of BLT1/BLT2 knockout in mouse RA models (68), a direct influence on joint tissues is plausible. In rat myoblasts, LTB_4 promoted proliferation and differentiation via BLT1 (171), contributing to muscle regeneration after injury. Both receptors are expressed in normal and OA subchondral osteoblasts (172) where they are involved in increased osteocalcin secretion associated with OA subchondral bone remodelling. Studies on OA chondrocytes indicate that the secretion of LTB_4 increase when COX-inhibitors are added to the culture (53), meriting further investigation of the influence of the LTB_4 system on chondrocytes.

Despite reports on chondrocytes expressing the 5-LOX and FLAP enzymes, which are necessary to synthesise LTB_4, and some potential effects on LTB_4 on chondrocyte functions (56), there are no previous reports on the LTB_4 receptors expression in human chondrocytes. In Paper I, we present novel data on both the high-affinity BLT1 receptor and the low-affinity BLT2 receptor being expressed in human cartilage and chondrocytes. In order to ascertain the role of these receptors we investigated readouts relevant to inflammation and cartilage biology. We were however, not able to confirm the upregulation of MMP-1 upon LTB_4 treatment of chondrocytes, which could be explained by the different time-frames of the treatments or different sensitivity of the assays (56). Jacob et al. described a favourable effect of the COX-2 dependent prostaglandin $F_2\alpha$ on both chondrogenesis and cartilage gene expression profile of the pellet cultures (173). We found no such effect of LTB_4 on chondrogenesis or gene expression profile, which can be explained by experimental differences, or that the lipid mediators are not comparable in this regard. The role of LTB_4 receptors in cartilage biology is thus still elusive and the protective effects of BLT1 or BLT2 knockout in murine studies, or effects of dual COX/5-LOX inhibition is most likely mediated through effects on the synovium and not cartilage.

A potential role of the receptors in cartilage, which was not framed by our experimental setting, is during fracture healing as suggested from studies showing that BLT1 is expressed in fracture callous chondrocytes (174). The currently available studies are restricted to murine experiments where LTB_4 production is limited through knockout of the 5-LOX or treatment with 5-LOX inhibitors, resulting in improved callous formation during early fracture healing (174,175). A recent study on mice investigating lipid mediators in callus after fracture reveal a rapid decline in LTB_4, suggesting a role of leukotrienes in mitigating the inflammatory response (176).

5.2 Paper II

Another molecule receiving attention in OA research is vitamin D. The role of vitamin D in regulation of bone health and serum calcium/phosphorous level is well described (177). The reason for the interest of vitamin D in the setting of OA originate from the discovery of extrarenal 1α-hydroxylase activity in inflammatory diseases like sarcoidosis, inflammatory bowel disease and rheumatoid arthritis (83,178), along with the notion of OA patients exhibiting a higher prevalence of vitamin D deficiency (179,180), although the latter is not confirmed in all studies (181,182). The link is

supported by a proposed relationship between vitamin D deficiency, low bone mineral density and OA (183,184), and an association to OA subchondral bone changes has been suggested (73,185). Since vitamin D has a low risk profile, it would be ethically acceptable to advise vitamin D supplementation as a preventive measure of OA development, given that a beneficial effect can be documented (26). Thus, great expectations have been placed in studies where OA patients were prescribed vitamin D supplementation, hoping to slow down the disease progression. Although pilot studies pointed to improved pain and function scores from vitamin D supplementation (75), the latest randomised trials found no effect of vitamin D supplementation on pain, function or cartilage volume (77,186). A recent review points to the heterogeneous and confusing outcomes in registry studies on vitamin D and osteoarthritis and highlight the lack of studies investigating vitamin D effects on articular cartilage degeneration and regeneration (187). As several external factors, like diet and sun exposure, are difficult to control for in observational studies, there is a need of *in vitro* experiments where these factors can be controlled.

Low amounts of VDR and 1α-hydroxylase have been reported in murine growth plate chondrocytes (188,189), while a handful of studies report on the expression of vitamin D receptor (VDR) in human OA and RA cartilage, and during chondrocyte culturing (81,82). The VDR seems to be absent or very modestly expressed in normal adult cartilage (123). During OA or RA development, and in hypertrophic chondrocytes, the receptor is detected (123,190). While 1α,25$(OH)_2D_3$ treatment of chondrocyte cultures is reported to increase secretion of MMP-1, -3, and -13 (190,191), the opposite is seen in synovial stromal cells (192) indicating a dual effect on joint tissues.

Since reports on VDR expression in cartilage tissue have been divergent (82,123), our initial efforts focused on studying the expression in our material. Only two previous reports from one group have reported on VDR expression in OA cartilage, and we have found no further studies from that group regarding this subject. The lack of staining of our OA tissue may be due to a random effect as our experiments were limited to four cartilage samples, that the chondrocytes in our samples were not in a hypertrophic state or that the VDR is absent or expressed below the detection threshold of our assay. Contrary to previous reports, we did not detect a regulatory effect of 1α,25$(OH)_2D_3$ on VDR gene- or protein expression (188,190), which could be explained by different timeframes of the experiments. We did however note that VDR expression increased upon treatment with IL-1β. Adding to the existing knowledge, we show that the receptor expression is rapidly repressed upon cell condensation with no staining of VDR already after 2 days of 3D culturing, illustrating that the expression of VDR relies on the differential status of the chondrocyte.

An autocrine/paracrine effect of vitamin D on cartilage would require the chondrocyte to exhibit 1α-hydroxylase activity as previously described in rat chondrocytes (188) and in human osteoblasts and synovial fluid macrophages (193,194). In Paper II, we provide novel evidence of 1α-hydroxylase expression in human cartilage, cultured chondrocytes and spheroids. The enzyme activity was supported by a dose-dependent increase in 1α,25$(OH)_2D_3$ levels in chondrocyte supernatants following treatment with 25$(OH)D_3$, as previously described in human osteoblasts (86).

Both 25$(OH)D_3$ and 1α,25$(OH)_2D_3$ have been shown to inhibit proliferation and induce differentiation in human osteoblasts (86), while proliferation of synovial stromal cells was inhibited by 1α,25$(OH)_2D_3$ (192). On the contrary, we found that both 25$(OH)D_3$ and 1α,25$(OH)_2D_3$ slightly increased

proliferation rates in chondrocytes, and had an unfavourable effect on the expression of matrix-associated genes. We also found that both $25(OH)D_3$ and $1\alpha,25(OH)_2D_3$ treatment in adherent cultures, decreased matrix production during chondrogenesis, which is not surprising given that vitamin D can be used to induce osteogenic differentiation (195). Considering the results from the recent studies on other joint tissues, vitamin D seems to have different effects on osteoblasts, synovial tissue and cartilage. Overall, it may be that vitamin D exert favourable effects in healthy stages, where receptor expression in cartilage is low or absent, while during disease conditions the effect on cartilage could be unfavourable.

5.3 Paper III

Patients that have suffered a localised cartilage injury or disease with subsequent debilitated knee joints can benefit from cell-based therapies that aim to repair the cartilage defect and prevent secondary OA. However, long term follow-up studies after ACI or microfracture indicate that up to 40% require further surgical treatment (161). Such observations indicate that cell-based therapies seem to benefit only a subset of patients. Through many years of *in vitro* studies with surplus ACI cells, we have observed that chondrocytes from different donors display different potentials to form spheroids. Donor to donor variation, and even variation between single cell clones from the same donor in chondrogenesis, has also been described by others (97). Both the hanging-drop and the pellet approaches to induce chondrogenesis rely on initial cell condensation. We therefore hypothesised that cell-surface receptor expression is of paramount relevance for chondrogenesis and that expression levels of these molecules could be used to select donors with increased chondrogenic potential. Based on previous publications, we selected a range of cell-surface markers, integrins, cell adhesion molecules and growth factor receptors that may be differentially expressed according to different chondrogenic abilities (97,109,196,197).

We utilised the Bern score to sort out donors displaying different chondrogenic abilities. The score has previously been used to compare chondrogenic capacity between different cell types (198,199), while the comparison of chondrogenesis in chondrocytes has been restricted to GAG measurements (97). The Bern score has been validated and found to correlate with GAG measurements in chondrocyte pellet cultures (152). In clinical studies, others have compared the expression of surface markers in transplanted cells with clinical outcomes (164). However, it is still unsolved whether the chondrogenic potency of cells, as checked in *in vitro* settings, correlates with clinical outcomes, and thus the extrapolation of results should be taken cautiously. From all the tested genes, the TGFBR3 transcript was the only marker differentially expressed between groups and inversely correlated with chondrogenic potential. This observation was unexpected and goes against previous observations on chondrogenesis in other cell systems (200,201). We did not validate our finding at the protein level and thus, interpretation of this result should be done with care.

Our study was designed in a similar way to the study of Grogan et al. (97), thus we were expecting to find comparable results in regards to the surface receptors that were investigated in both studies. The lack of confirmation by our study may be explained by the different cartilage sources as Grogan et al. used chondrocytes from autopsies. Furthermore, Grogan et al. divided their samples in two groups based on GAG measurements, although they published the paper on the Bern score one year ahead. Our results are thus more in line with the findings of Stenberg et al. (164) who detected no correlation between a predefined set of gene markers and clinical outcome.

Since the selected cell surface markers did not reveal any predictive value, we proceeded with a global protein analysis. Quantitative proteomics confirmed the results from gene expression since none of the selected cell surface markers were differentially expressed. Setting the significance level at 0.1, we found that the expression of the subunits of prolyl 4-hydroxylase, the enzyme that catalyse the formation of 4-hydroxyproline, a vital element in the triple helix formation of collagen (202), were upregulated in chondrocytes of high chondrogenic potential. In a previous study on chondrogenesis, the gene expression of the beta subunit was upregulated (203). Given its relevance in collagen biosynthesis, it is likely that this molecule represents a true biomarker of chondrogenic potential. However, since this is the result of a high-throughput assay, the findings needs to be validated by specific protein- and gene expression studies.

If future studies are able to detect molecular markers capable of stratifying patients that may have greater benefit of cell-based therapies, we are left with the question on how to utilise these markers. From a clinical point of view, having to decide the treatment based on cells harvested from cartilage biopsies is far more advanced and implies an extra procedure to the joint than measuring molecular markers in blood or even in synovial fluid. In recent efforts, a few markers with predictive potential in the clinics have been identified in synovial fluid samples (165,166). Probably, a combination of biomarkers with clinical characteristics of patients is perhaps the most viable option in regards to patient stratification.

As far as we know, no study has investigated whether chondrogenic potential *in vitro* is predicative of a favourable clinical outcome of ACI. To complement the knowledge gained in Paper III, we have recently obtained permission to compare the results of this *in vitro* study with the two-year follow-up clinical outcomes of patients from which the surplus chondrocytes were originally derived. This will allow us to validate whether the *in vitro* model for chondrogenesis, has predictive value in clinical settings.

6 Conclusion and implications

6.1 Paper I

In Paper I, we demonstrated that human articular chondrocytes express functional LTB_4 receptors and are thus susceptible for input in case of elevated levels of LTB_4 from inflammation or shunting after blocking the COX enzyme. We were however, not able to identify effects on chondrocyte biological functions *in vitro* as assessed by matrix production in spheroid cultures, regulation of cartilage signature gene expression and the release of matrix degrading enzymes or inflammatory cytokines. The LTB_4 axis has been suggested as a key player in inflammatory joint disease, and its direct effect on the recruitment of immune cells could play a major role, whereas direct LTB_4 effects on the cartilage tissue is less relevant. The role of LTB_4 receptors in cartilage thus remains elusive, although a recent report suggest that leukotrienes may hold an important role in the callous chondrocyte by resolving inflammation during early fracture healing.

6.2 Paper II

In Paper II, expression of VDR in cartilage at the protein level was elusive, but upregulated upon expansion in monolayer cell culture, and further enhanced under inflammatory conditions. In monolayer cultures both $25(OH)D_3$ and $1\alpha,25(OH)_2D_3$ induced enhanced proliferation while the effects on cartilage signature genes was unfavourable. The gene expression results were supported by the observations in chondrogenesis assay where GAG deposition and matrix production was decreased in chondrocytes treated with $25(OH)D_3$ or $1\alpha,25(OH)_2D_3$. We provide novel evidence of 1α-hydroxylase activity in chondrocytes, which could imply that chondrocytes contribute to the increased level of active hormone found in the synovial fluid of patients with inflammatory joint diseases. The overall impact of vitamin D on the chondrocyte seems to be less favourable in terms of cartilage healing. However, since the expression of receptors depends on differentiation status of the chondrocyte, the observed effects are probably more relevant in the diseased or inflamed joint.

6.3 Paper III

In paper III, we established that surplus chondrocytes of patients undergoing ACI have diverging potential of forming cartilage *in vitro*. Our data indicate that cell-surface molecules such as integrins and other cell adhesion receptors are not good predictors of the chondrogenic potential. High-throughput proteomic analyses uncover the prolyl 4-hydroxylase enzyme as a potential biomarker worth investigating in future studies along with the TGF-β receptor superfamily. In the near future, we plan to augment the data with the clinical outcomes of the ACI, which will enable us to investigate the correlation between clinical outcome and chondrogenesis. It has not been established yet if the chondrogenic potential of patient-derived chondrocytes *in vitro* correlates with clinical outcomes. We hope to clarify this matter in a future study where the data presented here are linked to the two-year follow-up clinical outcomes of operated patients.

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