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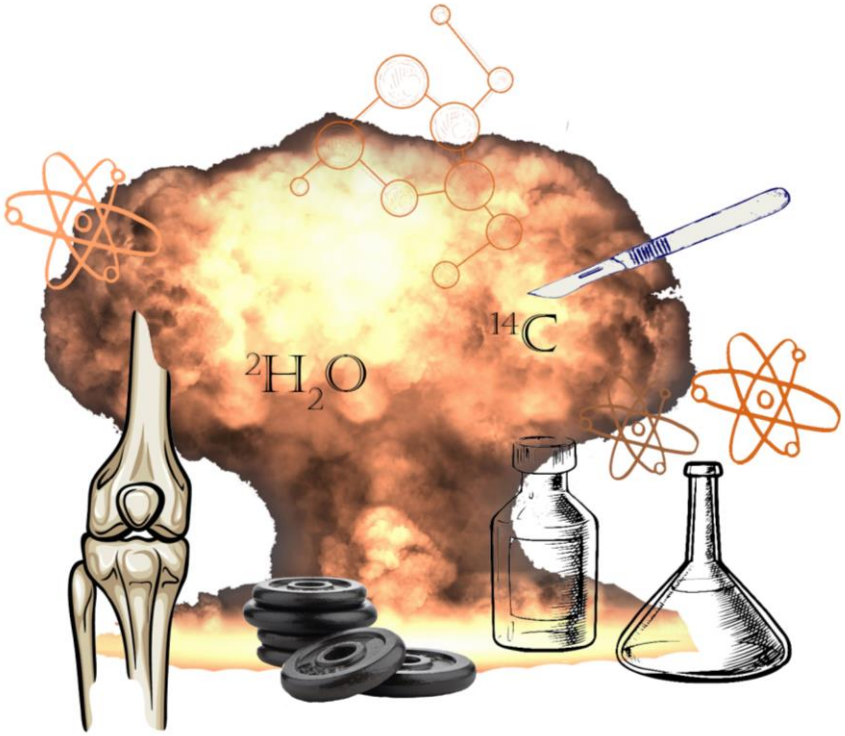

PhD Thesis

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Human cartilage growth, regional turnover *in vivo*, and the effect of exercise in late-stage knee osteoarthritis

Supervisors: Michael Kjær, Katja M. Heinemeier & Michael R. Krogsgaard

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Preface

The work presented in this PhD thesis are the result of my three-year research position as a PhD student at the Institute of Sports Medicine Copenhagen (ISMC). I am thankful for the received financial support for our project from The Danish Rheumatism Association. Further, I was supported by the Bispebjerg Hospital Research Grant and by the Center for Healthy Aging (CEHA) at University of Copenhagen funded by The Nordea Foundation in the Healthy Aging Grant. Finally, thanks to The Novo Nordisk Foundation, The Lundbeck Foundation, The VR Christiansen Travelling Scholarship, and to The Yde Foundation.

The thesis comprises scientific results in three studies denoted in roman numbers I-III. They were all conducted at the ISMC, Department of Orthopaedic Surgery M81, Bispebjerg and Frederiksberg Hospital, Denmark. Patients were recruited in collaboration with our sister department of Orthopaedic Surgery M50. During my change of research environment, I went to the University of Oxford at the Kennedy Institute of Rheumatology in professor Tonia Vincent's group. There, I learned a lot on the basic science of osteoarthritis; histology, cellular techniques, their limitations and possibilities, and this stay was indeed a great supplement to my clinical research. Unfortunately, the stay was shortened to the covid-19 pandemic, and no data from the stay is included in this thesis.

The manuscripts are enclosed at the end of the thesis.

Acknowledgements

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I will also thank the many colleagues at the ISMC. I have enjoyed your companionship and it has been a pleasure organising and being the host at the Christmas Lunches. A special thanks to my office buddies Anne Gylling, Anne-Sofie Agergaard, Cheng Zhang, Mads Bloch-Ibenfeldt, Annesofie Olesen, and Rikke Høfner. The atmosphere at the A-team-office has always been the best and the envy of the department. Also, thanks to Anja Jokipii-Utzon and Ann-Christina Reimann not only for your technical support in the lab, but also for great talks and jokes. You made pipetting tolerable, which is no easy feat. Finally, thanks to Maria Kjær and Else Pedersen for keeping the ISMC family together, for knowing every practical thing, and for always being willing to help. Finally, I feel very lucky for the colleagues Chloé Yeung, Antonios Giannopoulus and Cheng Zhang, who became my great friends. I will miss working with you, but I look forward to meeting you wherever place on the globe you might currently be.

I also need to say a big thank you to the orthopaedic surgeons in the knee-allo team; Jens Bagger, Mette Hornsleth, Morten Schmiegelow, Xiaodong Ma, Bent Olsen, and Wjeha Malik. Your enthusiasm, support, help with recruitment, and for giving me the tibia plateaus is much appreciated – I am happy to have worked as your colleague for half a year. Thank you to the nurses at Department M50, and especially Ingeborg Jørgensen for updating me on my participant's surgical schedule and making me and my research feel welcome in your busy day.

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Finally, thanks to all my participants. Without you, there would not have been any research.

Adam Jørgensen, August 13th, Copenhagen

Abbreviations

ACAN: Aggrecan
ACL: Anterior cruciate ligament
ADAMTS: A disintegrin and metalloproteinase with thrombospondin motifs
AGE: Advanced glycation end-products
ANOVA: Analysis of variance
APE: Atomic percentage excess
BMI: Body mass index
COL2A1: Collagen type II
COMP: Cartilage oligomeric protein
CS: Chondroitin sulphate
CTGF: Connective tissue growth factor
D2O: Deuterium oxide, $^{2}H_2O$ or heavy water
D-ala: ^{2}H-alanine
dGEMRIC: Delayed gadolinium enhanced MRI of cartilage
FGF-2: Fibroblast growth factor 2
GAG: Glycosaminoglycan
HA: Hyaluronic acid
HYP: Hydroxy-proline
IGF-1EA: Insulin-like growth factor 1
IL: Interleukin
KS: Keratan sulphate
MMP: Matrix metalloproteinase
MRI: Magnetic resonance imaging
mRNA: Messenger ribonucleic acid
OA: Osteoarthritis
PCL: Posterior cruciate ligament
PG: Proteoglycan
pMC: Percent modern carbon
PRG4: Proteoglycan-4 or lubricin
PTOA: Post-traumatic osteoarthritis
RCT: Randomised controlled trial
RT-PCR: Reverse transcriptase polymerase chain reaction
SD: Standard deviation
SEM: Standard error of the mean
SF: Synovial fluid
TGF-β: Transforming growth factor beta
TLR: Toll like receptor
TNF-α: Tumour necrosis factor alpha

Summary

Background: Articular cartilage is made for loaded movement of joints, and like other tissues of the musculo-skeletal system, cartilage can adapt to load with hypertrophy or atrophy primary by changing glycosaminoglycan content. Cartilage contains fibrillary collagen type II, which have been reported to have negligible renewability in adulthood. The joint disease osteoarthritis (OA) is characterised by cartilage loss, despite the fact that increased synthesis of aggrecan has been reported. Regarding collagen, the turnover in human OA are unclear, as studies use different techniques, species, and outcomes, yet, increased collagen synthesis is also widely reported. Nonetheless, as the matrix is failing in OA, the increased synthesis is either not reflected in the matrix content or increased breakdown outweighs the synthesis. In OA patients, regular physical activity has shown positive clinical effects upon pain and functional outcomes, but despite this fact, it is unknown how or if physical activity influences the cartilage physiology *per se*.

Aims: This thesis aimed at (1) determining if and when a cartilage growth pattern is present and (2) to determine the timing and duration of this growth in regional details. Further, in OA-affected cartilage from humans *in vivo* the aim (3) to characterise the regional collagen turnover and (4) the regional physiological effect of exercise.

Methods: Throughout this thesis, human knee cartilage from the tibia plateau was treated enzymatically to examine the stabile/insoluble fibrillary collagen type II. The growth pattern of cartilage collagen was determined using the bomb-pulse method, which relies on the rapid change in the atmospheric ^{14}C levels, and as synthesis of organic material incorporates carbon atoms, the atmospheric levels are then mirrored in the human tissue.

The regional turnover was assessed using deuterium oxide (heavy water, D_2O) as a tracer, administered orally by weekly boluses of initially 150 ml (70%) and three boluses of 50 ml (70%) in a total of four weeks. Cartilage from the medial tibia plateau/condyle was sampled centrally, under the meniscus, and from peripheral osteophytes, and were analysed for deuteriated alanine (D-ala) incorporation using mass spectrometry.

The regional effect of exercise was examined in a randomised controlled trial with acute loading (one-legged leg-press) prior to arthroplasty due to primary knee OA as the

intervention. Cartilage from the medial tibia condyle was sampled centrally, under the meniscus, and from peripheral osteophytes, and analysed for chondrocyte gene expression using real-time reverse transcriptase polymerase chain reaction.

Results: The enzymatic treatment resulted in a matrix containing collagen (~70%) and very low levels of GAGs (~1%). ^{14}C levels were different across each condyle with the oldest collagen centrally, meaning that central areas were made earlier in life compared to peripheral areas. Further, the medial condyle trended to have a concentric pattern towards the periphery (P = 0.056). The average age of cartilage collagen was 11.7 years ranging from 7–16 years, and the medial condyle was made slightly later than the lateral condyle at 11.4 versus 10.3 years.

The regional turnover of cartilage collagen matrix was similar centrally and under the meniscus showing D-ala of 0.063 % APE (atomic percent excess). Osteophytes showed higher turnover with D-ala of 0.072% APE. Compared to plasma proteins, the cartilage collagen turnover was lower, suggesting a restricted renewability capacity in late-stage human OA. The turnover in untreated cartilage was D-ala 0.075% APE and higher compared to the enzymatically treated cartilage at D-ala 0.065% APE. Thus, cartilage contains a fraction of proteins with higher turnover.

Acute loading increased TGF-β1 expression by 1.23-fold with no other effects. Regionally, increased expression of MMP-3, MMP-13, IGF-1Ea, and CTGF with a decreased expression of lubricin and COMP was found under the meniscus compared to central cartilage. Further, osteophyte cartilage showed different gene expression pattern compared to the proper articular cartilage with decreased expression of aggrecan, COMP, and FGF-2 with increased MMP-1, MMP-13, TGF-β3, and IGF-1Ea expression.

Conclusions: In conclusion, this thesis demonstrates that apart from osteophyte cartilage, the fibrillary collagen type II is initially made centrally and has very limited turnover in adulthood. Further, in late-stage OA, cartilage collagen has similar turnover despite regional differences in gene expression, and cartilage contains other matrix components with a higher turnover. Finally, in human late-stage OA, acute loading *in vivo* has minor effects on the cartilage.

Dansk resumé

Baggrund: Ledbrusken er lavet til ledbelastende bevægelser. Den kan tilpasse sig til forskellige grader af belastning med atrofi eller hypertrofi på samme måde som resten af bevægelsesapparatet, der primært sker ved at ændre indholdet af glykosaminoglykaner (GAG). Ledbrusk indeholder fibrillært kollagen type II, der har en negligibel fornyelseskapacitet i voksne. Ledsygdommen slidgigt er karakteriseret ved tab af brusk på trods af, at øget syntese af aggrecan er rapporteret. For kollagenomsætningen er der et uklart billede, da studier anvender forskellige teknikker, arter og typer af resultater, dog er øget kollagensyntese hyppigt er rapporteret.
Ikke desto mindre går ledbrusken til grunde ved slidgigt, så enten fører øget syntese ikke til mere kollagen, ellers er nedbrydningen større. I mennesker med slidgigt har regelmæssig fysisk aktivitet vist kliniske forbedringer på smerte og funktion, men på trods af dette faktum er det uvist, hvordan fysisk aktivitet påvirker ledbruskens faktiske fysiologi.

Formål: Denne afhandling har som formål (1) at undersøge om ledbrusken har et vækstmønster og (2) i så fald hvornår denne vækst er til stede i regionale detaljer. Desuden (3) at undersøge den regionale omsætning af ledbruskens kollagen ved slidgigt, og (4) undersøge den regionale fysiologiske effekt af belastning i ledbrusken fra patienter med sen-stadie slidgigt.

Metoder: Igennem denne afhandling har ledbrusken på tibia plateauet fra mennesker været behandlet med enzymer for at undersøge det stabile/fibrillære kollagen type II. Vækstmønsteret blev bestemt med bombe-puls-metoden, der afhænger af den hurtige ændring i atmosfærens ^{14}C niveau, og da syntese af organisk materiale indbygger kulstofatomer (karbon), er de atmosfæriske niveauer afspejlet i det menneskelige væv.

Den regionale omsætning blev undersøgt med deuterium oxid (tungt vand, D_2O) som sporstof, der blev drukket en gang om ugen i fire uger fordelt i portioner af 150 ml (70%) initialt og 50 ml de resterende tre uger. Ledbrusken på mediale tibiakondyl blev indsamlet centralt, under menisken og fra perifere randudbygninger (osteofytter), og ledbrusken blev undersøgt for indbygning af deuterium-mærket alanin (D-ala) ved brug af massespektrometri.

Den regionale effekt af belastning blev undersøgt i et randomiseret og kontrolleret forsøg med akut belastning (ensidigt benpres) som intervention før en ledudskiftende operation af

knæet på grund af primær slidgigt. Ledbrusken på mediale tibiakondyl blev indsamlet centralt, under menisken og fra perifere randudbygninger (osteofytter) og undersøgt for genekspression ved brug af "realtime" revers transkriptase polymerase kædereaktion.

Resultater: Den enzymatiske behandling førte til en matrix bestående af kollagen (~70%) og meget lavt GAG indhold (~1%). ^{14}C-niveauerne var forskellige på tværs af hver kondyl med det ældste kollagen centralt. Det betyder, at centrale områder bliver lavet tidligere i livet sammenlignet med perifere områder. Der var desuden en tendens til et specifikt koncentrisk vækstmønster (P = 0.056). Den gennemsnitlige alder af kollagen var 11,7 år i intervallet fra 7–16 år, og den mediale kondyl var færdig en smule senere end den laterale med 11.4 versus 10.3 år.

Den regionale omsætning af ledbrusken kollagen var ens centralt og under menisken med D-ala på 0.063 % atomprocent overskud (APE), mens ledbrusk fra osteofytterne havde højere omsætning med D-ala på 0.072 % APE. Sammenlignet med blodplasma-proteiner var kollagenomsætningen lavere, der tyder på en begrænset fornyelseskapacitet ved sen-stadie slidgigt hos mennesker. Omsætningen i ubehandlet ledbrusk var med D-ala på 0.075 % APE højere sammenlignet med det enzymbehandlede ledbrusk med D-ala på 0.065 % APE – derfor indeholder ledbrusk en fraktion af proteiner med en større omsætning end kollagen.

Akut belastning øgede TGF-β1 ekspressionen 1,23 gange uden andre effekter. Regionalt under menisken var der øget ekspression af MMP-3, MMP-13, IGF-1Ea og CTGF men nedsat ekspression af lubricin og COMP sammenlignet med den centrale region. Desuden havde ledbrusk fra osteofytter et andet ekspressionsmønster med øget ekspression af MMP-1, MMP-13, TGF-β3 og IGF-1Ea i kombination med nedsat ekspression af aggrecan, COMP og FGF-2 sammenlignet med den rigtige ledbrusk.

Konklusioner: Denne afhandling demonstrerer, at fraset ledbrusken på osteofytter, er fibrillært kollagen type II initialt lavet centralt med meget begrænset omsætning efterfølgende hos voksne. På trods af regionale forskelligheder i genekspression har ledbruskens kollagen ens omsætning, og ledbrusken indeholder desuden matrixproteiner med en større omsætning. Endelig har akut belastning *in vivo* kun mindre effekt på ledbrusken hos patienter med sen-stadie slidgigt.

1. Introduction and background

1.1 Articular cartilage is a specialised connective tissue made for loaded movement

In joints, hyaline cartilage covers the surface of bones. Articular cartilage contains 5% chondrocytes and 95% matrix. The matrix is comprised of a fibrillary collagen network embedded in a viscous gel. The gel contains water-attracting proteoglycan (PG) aggregates, and the fibrillary collagen retains the resulting swelling [1], while providing tensile strength [2,3]. The articular cartilage links with the subchondral bone below, and contains zones depending on depth due to the arcade orientation of the fibrillary collagen (superficial zone, transit zone & deep zone) [4]. Below the deep zone, a calcified fourth zone is found transitioning into the subchondral bone. When the cartilage is loaded, the compressed PGs disperses the force onto the rest of the joint surface thus decreasing the resulting pressure and stress [5], and transferring the load to the bone. The articular cartilage surfaces have a top layer containing the glycoprotein lubricin or proteoglycan-4 (PRG4) and the PG hyaluronic acid (HA). These components are produced by chondrocytes in the superficial cartilage layer and from synoviocytes to the synovial fluid (SF), and decreases the mechanical friction and sheer surface stress considerably [6,7]. Articular cartilage is thus made for loaded movement of joints. Similarly to other tissues of the musculo-skeletal system, cartilage can adapt to load with hypertrophy or atrophy [8].

1.2 Cartilage matrix components and their turnover/renewability

1.2.1 Proteo- and glycosaminoglycans

The large PG aggregates look like a bottlebrush. The long base is HA, which have attached around a hundred other PGs, mainly aggrecan [9]. Aggrecan is bound non-covalently to HA in one end and to the matrix in the other [9]. On aggrecan itself, the glycosaminoglycans (GAGs) keratan sulphate (KS) and chondroitin sulphate (CS) as well as other oligosaccharide chains are attached [10] (Fig. 1). In healthy cartilage, the half-life of full-size (i.e. complete length) aggrecan was estimated to be breakdown was 19.5, 23.5, or 87.9 years [11–13]. Thus, aggrecan is sequentially degraded. The major enzymes cleaving aggrecan are the matrix metalloproteinases (MMPs) and the real aggrecanases (*a disintegrin and metalloproteinase with thrombospondin motifs*, ADAMTS) [14]. They cleave at

different locations, and the resulting pieces (neoepitopes) either remains in the matrix attached or free-floating but unable to escape, or they are lost from the matrix into the SF [15]. Thus, pieces remaining in the matrix would still be functioning, but maybe to a lesser degree than the complete structure. Cleaving of aggrecan is initiated at the matrix-binding end and proceeds in a certain pattern [14,16], leading to declining CS content by this sequential removal (Fig. 1). The joint disease osteoarthritis (section 1.5) is characterised by cartilage loss, despite the fact that increased turnover [11] and increased aggrecan synthesis has been reported [17]. Nonetheless, as the matrix is failing in OA, this increased synthesis is either not reflected in the matrix content or increased breakdown outweighs the synthesis.

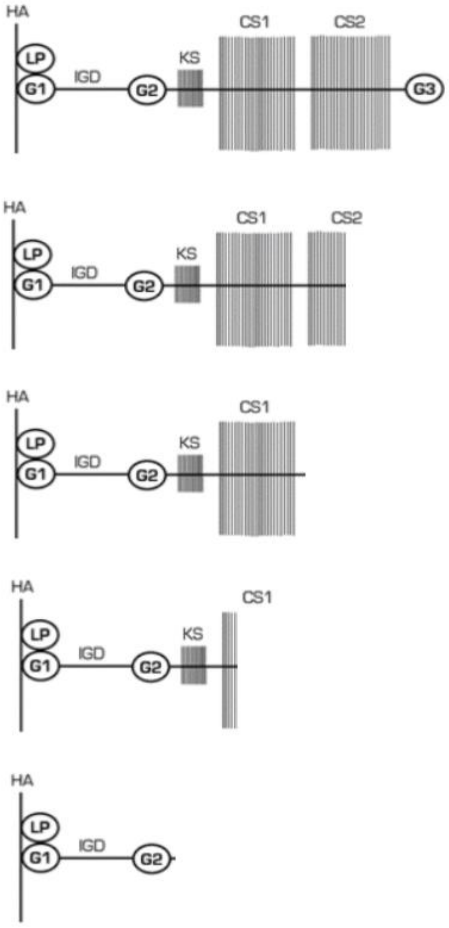


Figure 1: Aggrecan
Aggrecan has three globular domains (G1-G3), a KS rich zone, and two CS rich zones. Sequential cleaving leads to relatively more CS loss compared to KS. LP: link protein. IGD: Inter globular domain. The figure was modified from ref.11.

1.2.2 Collagen type II

In articular cartilage, the fibrillary collagen network represent around 60% of dry weight [1], primarily being composed of collagen type II (>90 %) [3]. The fibrillary network has a high tensile strength and low compliance due to enzymatic cross links [2,3]. Cartilage also contain minor quantities of collage type VI, IX and XI [9,18], which is further connected e.g. through biglycan and decorin [19]. The fibrillary collagen type II in cartilage is made from the coiling of three α-chains into a triple helix of procollagen, which is processed by N- and C-terminal cleaving and the subsequent covalent cross-linking into micro fibrils, which aggregates into larger fibrils and ultimately into fibres [20]. Cartilage tissue contains both soluble and insoluble collagen [21,22]. When procollagen molecules are initially produced, they are soluble but when they polymerize into collagen fibrils, they become cross-linked and consequently insoluble [20]. Thus, two fractions or pools of collagen type II exist in cartilage. Labelling of rats, has shown that most of the collagen is essentially inert in adult/mature rats and the dynamic components represent a minor fraction [23]. It seems

reasonable that a stable collagen matrix is required for normal organ structure and function, and thus this matrix would remain largely inert after completion [24], as shown in rats in different tissues most pronounced in the eye lens [25]. By enzymatic treatment of cartilage, this insoluble or stabile collagen fraction has been isolated and shown to have a half-life >100 years using rate of racemization of aspartic acid or accumulation of advanced glycation end-products (AGE) [26,27]. However, as these methods are influenced by physical and chemical factors i.e. pH and temperature [28,29], tracing incorporation of isotopes has been used, showing similar results in cartilage finding no major incorporation after maturity [30]. In contrast to the insoluble collagen, the soluble collagen type II (i.e. extractable by guanidine-HCl) has a reported half-life around seven years [13]. However, the soluble collagen fraction analysed was most likely only loosely associated to the fibrillary network [13]. Using a stronger acid or enzymatic digestion (as described above) could possibly provide a larger and but also more relevant fraction, i.e. incorporated but not stabile collagen type II.

With disease (i.e. osteoarthritis, see section 1.5), collagen turnover is less clear. A lack of any major turnover in mature/insoluble collagen was reported [30], nonetheless, short-term assessments have demonstrated increased synthesis [31–36]. These studies are using different techniques, species, and outcomes, and consequently, the collagen dynamics in human OA are unclear. Thus, long-term (but not life-long) direct measurements are needed.

1.3 Cartilage growth

As children become adults, their bones grow in length and diameter. The longitudinal growth is well characterised, initiated by cartilage expansion and ossification in the epiphysis and metaphysis until these fully mineralizes into bone, and the skeleton reaches maturity [37]. The articular cartilage never mineralizes and is made by different chondrocytes, however the articular cartilage growth and expansion laterally in the joint, during the skeletal growth is unclear [38]. The lateral cartilage expansion is vital for maintaining the cover as growth occurs, and a larger surface area limits the increasing pressure from the rising body weight as the individual grows. From animal models, the initial immature cartilage is exchanged during the skeletal growth before puberty with the exception of the superficial layer [39,40]. During puberty, the fibrillary collagen develops by changing the

orientation and enzymatic cross link content to become a mature/stabile collagen network [3,41,42]. In humans, similar studies are unavailable, however, if similar development is the case, then a minimum of new collagen is made in adulthood in the mature cartilage (see section 1.2.2). From our lab, earlier work assessed the mature collagen turnover by sampling centrally and peripherally on the tibia plateau with the purpose of comparing high vs low loaded areas [30]. An interesting finding was a positive correlation between the distance to any edge of the plateau and the corresponding age of formation, suggesting that central areas were older than peripheral. Further, when studying the supplemental data in detail, the difference in age of formation was less than five years, suggesting a very short time interval of cartilage growth. However, the sampling was made without any systematics regarding directional differences (anterior-posterior and medial-lateral) or differences whether sampling were from either of the two condyles. Thus, this interesting correlation needs a systematic verification across the entire plateau to determine if a growth pattern is indeed present and to determine the timing and duration of this period.

1.4 Loading affects the cartilage

Surrounding the chondrocyte, collagen type VI and the PG perlecan form a mesh-like capsular ultrastructure (the chondron) that modifies the mechanical load reaching each chondrocyte [43,44], thus allowing the chondrocyte to sense the cartilage load through the pericellular matrix [45]. Articular cartilage is made for loaded movement (section 1.1), and in humans, reduced loading has shown reduced cartilage thickness by magnetic resonance imaging (MRI) in knees of both paraplegic patients [46] and patients with an ankle fracture [47]. From animal models, histochemical analyses have been made [48]. Immobilisation of the knee joint led to reduced GAG content especially from the superficial zone [49,50] keeping the collagen content unchanged [49]. Cartilage without load was softer, resulting of either reduced collagen stiffness [51] or PG decrease [50]. In rats, MMPs and ADAMTS-5 were demonstrated to yield the atrophy by cartilage matrix degradation, however, passive movements on unloaded cartilage were preventive [52,53]. Restoring normal load does re-establish the stiffness [51] and GAG content [49,50], but only in the regions with actual weight bearing. Consequently, loading prevents cartilage thinning and softening, which could result in a cartilage being more susceptible to traumas, as collagen damage occurs earlier in thin cartilage [54]. Thus, the normal daily loading is necessary to prevent disuse atrophy in similar fashion the rest of the musculo-skeletal system.

Regular moderate loading generally leads to cartilage hypertrophy [8]. In animal models, moderate loading regimens in rabbits resulted in increased PG content in deep zone with unaltered collagen content [55], and in canines, moderate running led to increased PG in the transit and deep zone [56,57]. Human studies are less clear, as studies directly assessing a loading intervention shows different results. In adult women aged 20-40 years, moderate running for 10 weeks led to increasing GAG content assessed by delayed gadolinium-enhanced magnetic resonance imaging of cartilage (dGEMRIC) [58]. However, in post-menopausal women aged 45-55 years, moderate endurance or resistance exercise for 12 weeks led to no change of the cartilage [59]. In rodents, low and moderate intensity running increased lubricin gene expression after 4-8 weeks [60,61], which has been shown to protect chondrocytes from apoptosis [7]. In healthy human chondrocytes exposed to load, MMP-1 is either unchanged [62], or decreases together with MMP-3 and -13 due to increasing IL-4 [53,63,64], thus producing an anti-catabolic stimulus. In rats, cartilage IL-10 protein levels were found to increase with exercise [65]. In healthy human cartilage explants, the direct effect of IL-10 and IL-4 has shown both increased synthesis of aggrecan/PG [66,67] and collagen [68], but also no effect on either [69,70]. Thus, moderate load is widely considered beneficial for cartilage health and maintenance, by decreasing matrix breakdown and increasing GAG and lubricin content – albeit many uncertainties regarding the human *in vivo* response are present in this assumption.

Cartilage has a threshold for excessive mechanical load, and once this limit is surpassed the cartilage will initialize repair protocols [71]. Obesity, joint malalignment, abnormal gait, or joint instability (due to collagen diseases, meniscus or anterior cruciate ligament (ACL) tears) are conditions that already result in additional load on the cartilage. Thus, the presence of these factors will limit the additional load possible before reaching the threshold, and they have all be proven to be risk factors for developing OA [72]. Further, the threshold dose varies between *in vivo* and *in vitro* conditions [73]. From bovine explants, the degree of damage depends on the magnitude of load with the earliest damage resulting in softening, next adding loss of fibrillary cross links, and finally macroscopic collagen damage appears [74]. These *in vitro* data fit well with *in vivo* canine models, showing softening due to diminished GAG content both in superficial and transit zone [48]. Further, the superficial zone suffers a loss of collagen organization and cross links resulting in swelling from increased amount of water [75], and finally, subchondral bone

remodelling [76] with deep zone calcification/ossification [77]. Thus, overloading initiates loss of GAGs, which soften the cartilage matrix thus reducing the resistance capability, ultimately leading to more stress. Finally, the exposed superficial collagen is broken down, thus marking the irreversible transition to OA [78,79]. Irreversible, as in the collagen fibrillary network cannot be restored. This lack of renewability could be why articular cartilage with OA is unable to recover, though bearing in mind that increased synthesis of collagen (and the protective GAGs) has been reported (section 1.2). Thus, as the matrix turnover and the influence of load in humans *in vivo* is unclear, so is the potential for halting the gradually cartilage destruction seen in OA.

1.5 Osteoarthritis is a debilitating disease of the joint

With the destruction of the superficial fibrillary collagen, the loss propagates deeper. OA is characterised by increased catabolism, however, increased synthesis of matrix components is also seen in OA cartilage [80] (section 1.2). Thus, cartilage seems to counteract the destruction, but eventually this repair attempt fails [81], as the matrix breakdown products itself activate inflammation by toll like receptors (TLR)[82]. TLR increases the gene expression of pro inflammatory cytokines such as IL-1, -6, -8 and tumour necrosis factor α (TNF-α) as well as MMPs and ADAMTS, and the complement system ultimately resulting in even further breakdown and cartilage loss [78,83–86]. Thus, it seems that once the superficial collagen is lost, the development of clinical OA is eminent. OA is a whole joint disease characterised by pain and functional impairment (i.e. reduced range of motion and instability) [72,87,88]. There are different phenotypes of OA [89], but in this thesis, the focus is on the primary/idiopathic OA as classified by the American College of Rheumatology [90] and not e.g. post-traumatic OA (PTOA).

The treatment of OA is ultimately joint replacement surgery. This is an effective treatment, however, functional outcomes do vary and the prostheses' lifespan is limited [91,92], which is an increasing problem in an ageing world with further increasing expected life-span. Prior to surgery though, many none surgical treatments are recommended, including analgesics, weight loss, and exercise [93]. The clinical improvement by employing exercise therapy is well documented in countless trials, multiple reviews, and many systematic reviews with meta-analysis [94] – as encompassed in the creation of a systematic umbrella review [95]. However, despite this fact, the physiological effect on the

actual articular cartilage *per se* is unclear, as the only outcomes measured are either systemic biomarker studies or MRI of the cartilage, thus making it hard to make assumptions. In the biomarker studies, a systematic review showed reduced inflammation and matrix breakdown after months of exercise [96]. Few studies are available regarding exercise on OA cartilage. In middle aged women with mild knee OA, a one-year high-impact exercise intervention demonstrated improved GAG content of patellar cartilage by dGEMRIC [97] without any change in femoral nor tibia cartilage [98–100]. Thus, despite the established clinical improvement in OA from exercise, the actual effect on the cartilage is unclear at best due to the surrogate outcomes used (i.e. dGEMRIC and biomarkers).

2. Aims and hypotheses

From the knowledge presented in the background section, it is clear that OA and cartilage research still have areas of uncertainty. This study is composed of three individual studies, each having its specific aim as presented below:

- **Study I:** Here, the aim is to (1) determine if and when a cartilage growth pattern is present in healthy and OA diseased humans and (2) to determine the timing and duration of this growth in regional details. The hypothesis is that (1) a growth pattern on the tibia plateau does indeed exist during growth development (childhood and adolescence) with the central areas developed and that this occurs earlier than peripheral cartilage area, and (2) that the period of development is completed when reaching skeletal maturity.
- **Study II:** Here, the aim is to characterise the regional collagen turnover in OA-affected cartilage from humans *in vivo*. We hypothesise that a regionally difference in collagen turnover does exist across the joint surface.
- **Study III:** Finally, we aim to shed light on the regional physiological effect of exercise on the articular cartilage. We hypothesise that a response will be observed that includes (1) increased signalling for structural matrix components, and (2) stimulation of anti-inflammatory and anti-catabolic interleukin response in OA joint.

3. Methods and methodological considerations

In this section, a description of the study designs and methods used is presented (table 1). Methodological considerations are reported within the relevant method section.

Table 1: Overview of studies included in the thesis

Parameter	Study I	Study II	Study III
Study design	Observational	Prospective cohort	Randomised controlled trial
Study duration	Life-long	Four weeks	One day
Intervention	—	D2O ingestion	Acute loading (using resistance exercise)
Measurements	^{14}C levels	D-alanine, gene expression	Gene expression, GAG immunoblotting
Main outcome	Growth pattern	Regional turnover	Regional loading effect
Plateaus used:			
Healthy	n=2	—	—
Primary OA	n=2	n=20	n=32
Post-traumatic OA	n=2	—	—
Sampling region:			
Central region	Yes	Yes	Yes
Submeniscal region	Yes	Yes	Yes
Osteophytes	—	Yes	Yes

D2O: Deuterium oxide. D-alanine: Deuteriated (^{2}H) alanine.

3.1 Study designs

The three studies were performed at The Institute of Sports Medicine Copenhagen at Bispebjerg Hospital, however, healthy tibia plateaus were supplied from the musculoskeletal tumor section at the department of orthopaedic surgery, Rigshospitalet, Copenhagen, Denmark. The trial resulting in study II and III recruited patients for participation at Bispebjerg Hospital from the outpatient clinic of the orthopaedic department. Patients with OA of the knee as classified by the American College of Rheumatology [90] scheduled for a knee replacement surgery by an orthopaedic surgeon

were considered for participation. The trial leading to study II and III was registered on ClinicalTrials.gov (NCT03410745). All participants gave written informed content and the studies were approved by the Ethical Committee of the Capital Region of Denmark in accordance with the Helsinki Declaration II.

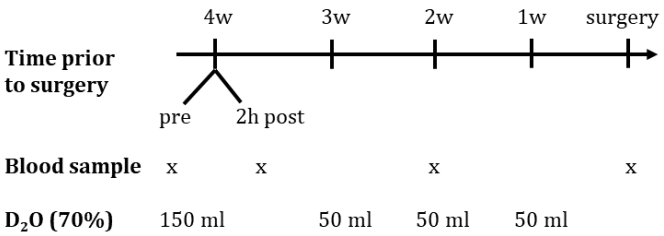


Figure 2: Schematic representation of protocol for study II
The study protocol is initiated four weeks (4w) prior to the scheduled knee replacement surgery. The blood samples are drawn before administering deuteriated water at 70% atom concentration.

In brief, study I used previously collected tibia plateaus with systematically sampling of biopsies, which were treated enzymatically before measuring ^{14}C levels. In study II, weekly oral boluses of deuterium oxide were ingested and traced in blood samples starting four weeks prior to arthroplasty (Fig. 2). The tissue was treated enzymatically to isolate collagen and analysed for regional deuterium-labelled alanine (D-ala). Study III was a one-day two-arm randomised controlled trial using an acute loading/resistance exercise intervention compared to usual care (sitting down reading or watching television) prior to knee replacement surgery. The cartilage was analysed for the regional effect on gene expression. Participants were randomly allocated 1:1 to one of the two groups using the minimisation software MinimPy version 0.3 (Python Software Foundation, Beaverton, OR, USA) [101], stratified by sex, age, and BMI.

Study II and III included eligible patients being 50–90 years old, having primary OA, a body mass index (BMI) within 18.5–40 kg/m², and being non-smoker. Patients were excluded if they had other diseases of the joint (rheumatoid arthritis (RA), gout, or psoriasis arthritis), systemic inflammatory diseases, diabetes, or cancer, participated in regular strenuous exercise, had oral ingestion or intra-articular injection of corticoid-steroids within the last three months, or required an interpreter. For study II, patients should be tracer naïve, and willing to schedule the surgery later than four weeks (the treatment guarantee is one month in Denmark) to allow time to complete the study protocol (Fig 2). For study III, patients were excluded if they used non-steroid anti-inflammatory drugs (NSAIDs) regularly within the last three months.

3.2 Tissue collections

In study I, cartilage from previously collected tibia plateaus [30] were used. Only those containing sufficient cartilage to perform the desired sampling were selected, i.e. only minor tissue sampling already performed and the plateau appearing without areas of complete erosions. Healthy cartilage plateaus were from surgical waste from distal femoral osteosarcomas not influencing the tibia cartilage. Four plateaus of OA cartilage were from total knee replacement surgery due to OA. To standardize sampling, a template was used to obtain eight cylindrical full depth cartilage biopsies systematically across the entire plateau with additional eight samples from the larger medial condyle to enhance details in the anterior to posterior direction, leading to a total of 12 from the medial and four from the lateral condyle (Fig. 3).

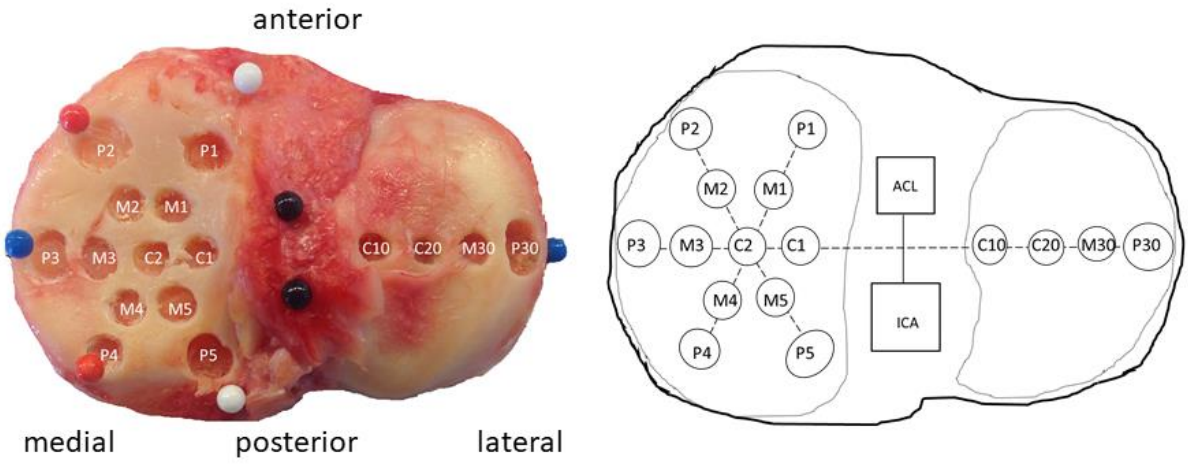


Figure 3: Systematic biopsy sampling

Left: Photograph of a right tibia plateau after sampling of cartilage tissue. **Right:** Schematic representation of the same plateau. Biopsies were sampled by locating the anterior cruciate ligament (ACL) and the intercondylar area (ICA) (black pins) marking the anterior-posterior line. A perpendicular line was made giving the longest possible span across (blue pins). Four samples were collected from the most central (C1 & C10) and peripheral (P3 & P30) location possible on each side, and two additional samples evenly distributed in between, both medially (C2 & M3) and laterally (C20 & M30). On the medial condyle, we located the points where the cartilage tissue curved from the central to anterior/posterior side near the horns of the medial meniscus (white pins). Two lines between the white pins and sample C2 were drawn and biopsies were taken the most peripheral location possible (P1 & P5) and another evenly between these and C2 (M1 & M5). Two bisecting lines between samples P1–C2 and C2–P3 and between P5–C2 and C2–P3 were marked (red pins). Biopsies were taken the most peripheral location possible (P2 & P4) with another evenly distributed between these and C2 (M2 & M4). This led to systematic sampling of 12 samples medially and four laterally.

In study II and III, the leftover tibia plateaus were collected during surgery and immediately transferred to the laboratory on ice. From the medial tibia condyle, several full-thickness cartilage biopsies were sampled regionally, i.e. centrally and under the meniscus. By cutting with a scalpel, the subchondral bone was removed from biopsies. From osteophytes (if present), slices were sampled using a scalpel. All cartilage for messenger ribonucleic acid (mRNA) extraction was snap-frozen in liquid nitrogen and kept on −80°C until further analysis, while cartilage for isotope analysis was weighted, cut perpendicular to the surface, freeze-dried, and weighted again. Blood samples were collected in K_3-EDTA plasma tubes (Vacuette, Greiner Bio-One, Kremsmünster, Austria), rested 30 minutes on ice, and centrifuged for 10 minutes at 4°C at 3172 G before the plasma fraction was collected and kept on −80°C until further analysis.

3.3 Enzymatic treatments

To measure the collagen component of the cartilage matrix (section 1.2.2), enzymatic treatments were needed to isolate this fraction of interest. In brief, we used hyaluronidase for GAG removal in raw (but freeze-dried) cartilage, trypsin for removal of non-fibrillary collagen and other smaller molecules, and papain for the complete digestion of non-hydrolysed cartilage to perform assays for GAGs and hydroxy-proline (HYP).

3.3.1 Collagen purification

By using enzymatic treatment, the GAGs can be removed with minimal collagen loss [102]. Freeze-dried samples were treated overnight with hyaluronidase (H3506, Sigma) [5 U/ml in 0.05 M sodium acetate and 0.15 M NaCl (pH=6)] at 37°C. The samples were centrifuged, had the supernatant (containing the GAGs) removed, before the cartilage slices were washed with isotonic NaCl. Trypsin is not able to cleave native triple helical collagen [103], and an indigestible cartilage matrix of predominately fibrillary collagen type II would therefore remain. Trypsin (T8802, Sigma) [1 mg/ml in PBS] was added and samples were incubated at 37°C overnight. Samples were centrifuged, washed with PBS, 0.7 M KCl, and distilled water before being freeze-dried, weighed, and kept at −80°C for later analyses.

3.3.2 Papain treatment

To verify the extraction of collagen and removal of GAGs, assays detecting HYP and GAGs were performed. The cartilage samples were incubated at 60°C overnight with papain (P3125, Sigma) [papain (0.125 mg/ml) in 100 mM sodium phosphate buffer, 10 mM Na_2-EDTA, and 10 mM L-cysteine (pH 6.5)]. This results in a complete digestion of the cartilage.

3.4 Biochemical analyses

3.4.1 Glycosaminoglycan assay

For GAG quantification, the diluted papain digest was mixed with 1,9-dimethylmethylene blue (DMMB) solution [38 µM DMMB in 40 mM NaCl, 40 mM glycine, pH=3], and absorbance were read at 595 nm and 540 nm wavelengths (subtracted) and compared to a known standard curve of chondroitin sulphate C (C4384, Sigma, Darmstadt, Germany).

3.4.2 Glycosaminoglycan immunoblotting

The hyaluronidase-extracted GAG fraction was analysed using immuno-assays of antibodies visualised by chemo-luminescence. Duplicates was dotted on a membrane (Hybond N+, Amersham, Brøndby, Denmark) and incubated in blocking buffer [5% skimmed milk powder in tris-buffered saline (TBS) with 0.05% Tween20 (TBS-T) (Sigma, Darmstadt, Germany)] for 1 hour at 20°C with diluted primary antibodies [chondroitin sulphate (Sigma, Brøndby, Denmark), keratan sulphate (US-Biologicals, Salem, MA, US), and hyaluronic acid (Bio-Rad Laboratories, Hercules, CA, USA)]. Next, the membranes were washed in TBS-T, before incubation with horseradish peroxidase-conjugated secondary antibodies [anti-mouse and anti-goat (DAKO, Glostrup, Denmark)] diluted in blocking buffer (TBS-T) for 1 hour at 20°C. Membranes were washed with TBS-T and TBS, before incubation with SuperSignal Femto Reagent (Pierce, Thermo Fischer Scientific, Waltham, MA, USA) for visualisation and imaged by CCD camera (Bio-Rad Laboratories, Hercules, CA, USA). Chemiluminescence was quantified with densitometry after normalizing to background with ImageJ software, and normalised to initial dry weight of the cartilage sample.

3.4.3 Hydroxy-proline assay

Here, HYP content was used as a proxy for the total collagen content. The papain digest was hydrolysed in 6 M HCl overnight at 110°C, dried, rehydrated with distilled water, and dried again before adding an acetate-citrate buffer (0.6% acetic acid, 130 mM citric acid, 440 mM sodium acetate, 425 mM NaOH, pH=6). A chloramine-T solution (60 mM chloramine-T, 50% 1-propanol was added, and samples incubated at room temperature for 20 minutes. Next, an aldehyde perchloric acid solution [1 M 4-dimethylaminobenzaldehyde, 60% 1-propanol, 22% perchloric acid (70–72%)] was added and incubated for 25 minutes at 60°C before the reaction was stopped in an ice-bath. The samples were read at 570 nm wavelength and compared to a known standard curve of hydroxyproline (H1637, Sigma, Darmstadt, Germany). As collagen has been found to contain around 13.4% HYP, the concentrations were converted to collagen concentration by multiplying with a factor of 7.5 [104].

The HYP assay is limited by the fact that the change in colour is not specific for HYP, and further, has an influence of time. However, if the sample is pure, the colour change would provide an accurate estimate as only HYP is found in collagen, and by using the mean of duplicates, or better yet using the median of triplicates, the effect of time will be mitigated. In the lab, small amounts of cobber accidently went into the samples during preliminary tests and coloured the samples yellow. This had a minor effect on the HYP measurement, however, if a larger amount of cobber was added deliberately, substantial changed in the HYP content was observed. Finally, the conversion factor has not been determined in human cartilage but relies on measurements of other collagenous tissues (e.g. tendons).

3.5 Measurements of isotopes

In this thesis, we have used isotopes to examine the growth and turnover of the cartilage matrix. In study I, we took advantage of the so-called bomb-pulse method, and in study II, we traced orally ingested deuterium into the matrix.

3.5.1 The bomb-pulse

In the years 1955–63, a major rise in atmospheric ^{14}C levels happened due to testing of nuclear bombs. After the Partial Test Ban Treaty in 1963, levels dropped and left a spike, which is known

as the bomb-pulse (see manuscript for study I, figure 3). Plants incorporate ^{14}C from atmospheric $^{14}CO_2$, and by consuming these plants, ^{14}C accumulates in growing organic tissue from animals and humans. The turnover rate of tissues determines the level of incorporation, and tissues with rapid turnover e.g. muscle have a content of ^{14}C equally to the current level in the atmosphere [105]. Instead, inert tissues of predominantly collagen (section 1.2.2) e.g. the eye lens has ^{14}C levels equal to the atmospheric levels, when this tissue was made i.e. had high turnover [106]. The tissue dynamics combined with the pulse in ^{14}C levels, allows a determination of human tissue turnover from individuals born in the years spanning the pulse.

In study I, comparing the ^{14}C levels in the cartilage biopsy with the historical atmospheric ^{14}C levels [107,108] allows for an estimate of the year of tissue formation. By comparing this year and the donor's birth year, the donor's age at the time of cartilage tissue formation is calculated. However, as the pMC is a mass weighted average of the ^{14}C levels accumulated during growth, this method is unable to say that the entire sample was made during the particular year. Thus, the actual tissue formation could very well have happened years before and after. Nonetheless, this method measures the life-long turnover from embryogenesis to tissue sampling, and as mentioned (section 1.3), other measurements are affected by temperature and pH, which is not the case for the bomb pulse.

Cartilage samples were analysed for ^{13}C, ^{14}C, and ^{15}N isotopes as described elsewhere [109]. The radiocarbon dating results are reported according to international convention [110] and ^{14}C content are given as percent modern carbon (pMC) based on the measured $^{14}C/^{13}C$ ratio corrected for the natural isotopic fractionation by normalizing the result to the standard $\delta^{13}C$ value of −25‰ VPDB (Vienna Pee Dee Belemnite: a $\delta^{13}C$ calibration standard.

3.5.2 Heavy water, deuterium oxide

Heavy water or deuterium oxide, D_2O (^2H_2O 99.8% Cambridge Isotope Laboratories Inc., Andover, MA, USA) was used as a tracer. Participants orally ingested boluses containing 70% D2O, initially 150 ml and subsequently 50 ml for the remaining three boluses using recommended approaches[111,112]. By reversible transamination, the D_2O will label the amino acid alanine by deuterium on four carbon positions, thus providing up to fourfold magnification for bound deuterium enrichment compared to other amino acids [113]. Further, alanine has been shown to be

consistently labelled [114] and more closely associated with the concentration of heavy water [115]. The deuterium-labelled alanine can then be measured in proteins, if alanine is incorporated by synthesis *de novo*. Other labelled amino acids could have been used instead e.g. ^{13}C-proline, ^{13}C-leucine, or ^{13}C-phenylalanine, which are all commonly used. However, these tracers are costly, only allows measurements over durations of up to 8–12 hours, and require preparation of sterile infusions, venous/arterial cannulations, and multiple biopsy collection, all within a controlled laboratory environment[116]. Finally, as low levels of enrichment were expected, the longest labelling duration logistically possible was prioritised, and thus, analysis on alanine was chosen.

The analyses were performed as described elsewhere [114]. In brief, precipitated plasma proteins and enzymatic treated cartilage samples were hydrolysed in 6M HCl at 110°C overnight to liberate the amino acids. Plasma samples for free amino acids were acidified, purified on cation exchange resin columns (Bio-Rad, Hercules, CA, USA), liberated using 2M NH_4OH for alkalinisation, and dried under N_2 flow at 70°C. Finally, the amino acids were derivatised to their corresponding N-acetyl-N-propyl (NAP) esters for analyses. Protein incorporation was analysed using gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS) and free plasma enrichment was analysed by liquid chromatography isotope ratio mass spectrometry (LC-MS/MS) systems (Finnigan, Bremen, Germany). The $^2H/^1H$ ratio was converted to atom percent excess (APE) as described elsewhere [116].

3.5.3 Analysis on soluble collagen

Collagen exists in a soluble and insoluble fraction (section 1.2.2). In cartilage, the PG and GAGs have been shown to have a high turnover, as does the soluble collagen albeit to a lesser degree. Soluble collagen very loosely attached to the fibrillary matrix have been extracted using guanidine-HCl [13], but a stronger acid or trypsin treatment would likely yield a soluble collagen type II fraction loosely incorporated into the actual matrix. This fraction could possibly maintain the fibrillary collagen, thus limiting the damage leading to OA (section 1.5). Initially, study II was created to examine the turnover of this soluble collagen fraction as well. Mass spectrometry requires the full separation of cartilage fractions before amino acid separation by gas chromatography as the following combustion results in the complete breakdown of amino acids into N_2, CO_2, and H_2, where the isotope ratio (i.e. $^2H/^1H$) is measured by mass spectrometry. Thus, the cartilage had to

be separated into fractions by treatment to get an accurate measurement of deuterium incorporated alanine using GS-C-IRMS.

Initially, extraction on raw cartilage by 0.5 M acetic acid did not extract any collagen measured by HYP assay. Similar result was obtained by treating the raw cartilage with hyaluronidase prior to acetic acid extraction. Thus, sequential enzymatic treatment by hyaluronidase to remove GAGs, and then trypsin to extract the soluble but loosely incorporated collagen fraction was used. This trypsin digest did contain collagen as determined by HYP assay. However, as measurements were planned to be made on alanine (section 3.5.2), the large amount of alanine in trypsin would influence our measurements, and thus, trypsin needed to be removed before analysis.

The trypsin digest was spun through filters (30 kDa cut-off) and rinsed using PBS. The filter content was resuspended in PBS but showed no collagen by HYP assay. This was unexpected, as collagen fibrils are around 200 kDa in size, whereas trypsin is 23 kDa before it cleaves itself. Further, it seemed that the filtration itself affected the results found in the HYP assay. Therefore, filtration was forfeited. Instead, collagen precipitation of the trypsin digest using high NaCl (2.5 M) was attempted. The samples were stirred for 24 hours at 4°C and centrifuged at 18.200 g for 2 hours. The supernatant was removed, and the (invisible) pellet was hydrolysed and analysed by HYP assay. Unfortunately, no collagen was detected in the pellet. Therefore, the supernatant was hydrolysed and analysed by HYP assay, however, by adding 6 M HCl to the already 2.5 M NaCl, precipitation occurred. The results on HYP assay were inconclusive, possibly due to the precipitation. Thus, a few samples of the (invisible) pellet resulting from centrifugation were analysed using mass spectrometry. This showed that no collagen remained, and thus, separation of trypsin and collagen was unattained. Thus, measurements on the trypsin-digestible fraction were forfeited.

3.6 Exercise intervention

The exercise intervention was supervised by a medical doctor and began by a light warm-up on an ergometer bike for 5–10 minutes. In a leg-press machine (Techno Gym, Cesena, Italy), participants were seated upright with the hip bended 90°. To ensure load on the knee of interest only, one-legged exercise was performed. The exercising leg extended from 100° to near fully stretched

against a vertical plate, while the other leg rested on a horizontal plate. Due to safety concerns of the overnight fasted participants with knee pain, we performed a 5 repetition maximum (RM) test as an indirect measure of maximum strength to calculate the corresponding 1 RM [117]. Three sets of eight repetitions at 70% 1 RM and three sets of four repetitions at 80% 1 RM were performed at controlled speed.

Initially, the study design contained a twice a week exercise intervention for four weeks in combination to heavy water ingestion. Our initial program would have progressed in load beginning with 12 repetitions at 60% 1 RM and 8 repetitions at 70% 1 RM, increasing to the final intensity on the day of surgery of eight repetitions at 70% 1 RM and 4 repetitions at 80% 1 RM. However, of great surprise, only one patient would participate in the trial, and several patients declined to participate unless randomised to the control group. Thus, the exercise intervention was removed from the four weeks labelling study. Instead, study III used acute exercise intervention on the day of surgery (without labelling) to examine the effect of exercise. The final intensity was used to ensure sufficient load on the OA cartilage.

3.7 Gene expression of mRNA

In brief, RNA was extracted from cartilage and osteophyte tissue by homogenisation in a guanidine thiocyanate/phenol solution [TriReagent (Molecular Research Center, Cincinnati, OH, USA)]. Next, the homogenate was separated using bromo-chloropropane (Sigma, Darmstadt, Germany) and the water phase containing RNA precipitated by a poly-acryl carrier and isopropanol. It was then further extracted using the RNeasy MinElute Cleanup Kit (Qiagen, Hilden, Germany) as per manufacture's protocol. The RNA concentration was determined by the RiboGreen assay (Molecular Probes Inc., Eugene, OR, USA). The isolated RNA was reversed transcribed to complementary deoxyribonucleic acid (cDNA) using poly-dT (Invitrogen, Nærum, Denmark) with the OmniScript Kit (Qiagen, Hilden, Germany) as per manufacture's protocol. Target mRNAs were quantified using real-time reverse transcriptase polymerase chain reaction (Stratagene, La Jolla, CA, USA) using cycle threshold (Ct) values from a standard curve of DNA oligos (Ultramer, Integrated DNA Technologies Inc., Leuven, Belgium) with a DNA sequence corresponding to the expected PCR product. The specificity of the PCR products was confirmed by melt curves of unknown samples and known oligos. Expressed values are normalised to internal

control of large ribosomal protein P0 (RPLP0), and further validated using glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

3.8 Statistics

In study I, statistical analyses were performed using GraphPad Prism version 8.4.1 (GraphPad Software, San Diego, CA, USA). Repeated measures one-way analysis of variance (ANOVA) or mixed-effects analysis (if values were missing) with the Geisser-Greenhouse correction for sphericity were used. Within areas of each condyle, Holm-Sidak's multiple comparisons test was used post hoc. Friedman test was used for differences in the ranking order of growth with Dunn's multiple comparisons test post hoc within areas on the medial condyle. Paired t-test was used for difference between medial and lateral condyles.

Study II also used GraphPad Prism for analyses. The osteophyte data had different variation compared to proper articular cartilage, thus, differences in cartilage by location were assessed using Friedman test with Dunn's multiple comparisons test post hoc. For difference between untreated and treated samples, Wilcoxon t-test was used. RNA targets were analysed by paired t-test on logarithmic values.

In study III, statistical analyses on gene expression data were performed using SigmaPlot version 13.0 (Systat Software Inc., San Jose, CA, USA), while GAG data were analysed in GraphPad Prism. Baseline results were compared using Mann-Whitney test. Effects of exercise intervention and regional location were analysed using repeated-measures two-way ANOVA with Tukey's multiple comparisons test post hoc. However, as GAG extractability data contained missing values, the equivalent mixed-effects analysis was used instead with Holm-Sidak's multiple comparisons test post hoc.

Throughout this thesis, statistical significance was defined as P-values < 0.05 for all analyses. Depending on distribution, the data are presented with the aim of using means ± SD (or 95% CI) to show the spread of the data distribution i.e. the uncertainty of each data point, while using either (geometric) means/medians with SEM/interquartile range to show the uncertainty on a mean/median value when comparing groups.

4. Results and discussion

In this thesis, the articular cartilage of the human tibia in knee was examined to (1) characterise the development, growth, and life-long turnover, (2) determine the regional collagen turnover in late-stage OA, and (3) examine the regional influence of exercise in late-stage OA.

4.1 Study I: Collagen growth pattern

In 16 samples across the entire tibial condyle, we applied the bombe-pulse method to trace the life-long incorporation of ^{14}C in enzymatically treated cartilage to characterise the developing growth pattern and timing of cartilage collagen.

4.1.1 Participant characteristics

As every donor were born just after the peak of the pulse, they solely experienced declining levels of ^{14}C throughout their life, and therefore, high levels of ^{14}C means that the matrix contains remnants of older levels, and consequently made early in life (section 3.5.1). In total, six plateaus were used (table 2).

Table 2: Donor characteristics.

Donor	Birth year	Tissue type	Tibia plateau cartilage condition
#1 male	1971	Healthy cartilage	Normal. No sign of OA.
#2 male	1971	Healthy cartilage	Normal. No sign of OA.
#3 female	1968	Primary OA	Moderate medial and lateral OA.
#4 female	1967	Primary OA	Moderate medial and lateral OA.
#5 female	1967	Post-traumatic OA	Moderate medial and lateral OA. Medial meniscus lesion in the twenties (specific age unknown).
#6 male	1967	Post-traumatic OA	Moderate medial and lateral OA. Severe patello-femoral OA. Patella fracture and posterior cruciate ligament rupture at age 30.

4.1.2 Growth pattern

The isotope measurements were made on the enzymatic treated cartilage samples, which had a high content of collagen (697.7 µg/mg dry weight ±111.9 SD) and negligible GAG content (10.7 µg/mg dry weight ±2.8 SD) regardless of tibia location. Hence, the data presented on the trypsin-resistant matrix are largely representative of fibrillar collagen (section 3.3).

The measured levels of ^{14}C in percent modern carbon (pMC) are presented for every location for each donor (Fig. 4A) with a significant difference across the plateau (P = 0.049). The medial condyle had eight additional samples taken and therefore the means from every area were calculated and presented (Fig. 4B). Here, differences in areas showed a solid trend (P = 0.056) and a significant linear trend test (P = 0.007). From the presented data, it is clear that donor #6 demonstrated opposite growth pattern, and if this dataset was excluded from the analysis, then the trend became statistically significant (P = 0.001) with post hoc differences between every area i.e. C1-C2 vs. M1-M5 (P = 0.023) and M1-M5 vs. P1-P5 (P = 0.005). To take the fluctuations of pMC levels into account, a possible order of growth was examined by ranking every sample location of each condyle, i.e. 1–12 medially and 1–4 laterally. If similar pMC levels were found in different locations, the mean rank was used for the locations. The median rank for each location is presented with 1 developed earliest and 12 latest in life for each donor (Fig. 4C). Again, a significant difference across the plateau was found (P = 0.015), and when combining the mean ranks of the areas on the medial condyle, a trend was found (P = 0.072) (Fig. 4D). Nevertheless, if donor #6 with opposite growth were to be removed from the analysis, significance was achieved (P = 0.0008) with the addition of a difference between the central and peripheral area (P = 0.0047) post hoc. Finally, an illustration of the growth pattern on the plateau is presented schematically (Fig. 4E).

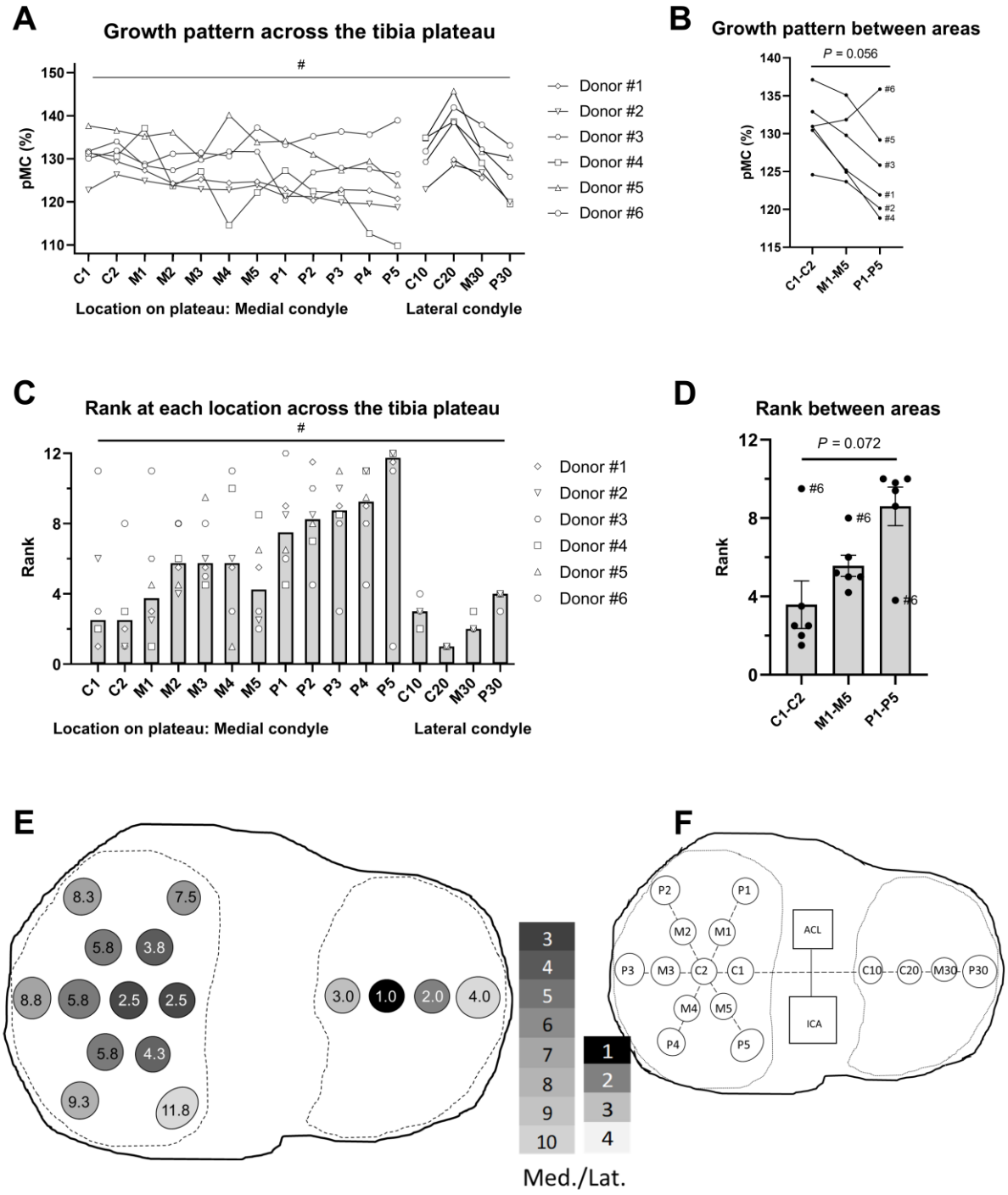


Figure 4: Cartilage growth pattern

A: Tibia plateau growth pattern. Values are individual pMC levels. **B:** Growth pattern between central (C1-C2), middle (M1-M5), and peripheral (P1-P5) areas on the medial condyle. Values are mean pMC levels for each donor. **C:** The rank at each location. Values are individual ranks, and bars represent median rank for each location. **D:** The mean ranks between central (C1-C2), middle (M1-M5), and peripheral (P1-P5) areas on the medial condyle. Values are mean ranks for each donor, and error bars represent mean rank for each area ±SEM. **E:** Schematic presentation of the growth pattern on the tibia plateau. Values are median ranks at each location. **F:** Location legend. ACL: Anterior cruciate ligament. ICA: Inter condylar area. # equals $P < 0.05$ for analysis.

As presented in section 1.3, a positive correlation was detected between corresponding age of formation and distance to the edge of the tibia plateau [30]. Now, the current results can not only verify this correlation, but also suggest that a central location on each condyle is the developing point for radial growth. Due to the limited number of donors with one donor showing outlying data, further statistical significance was unattainable. Yet, even with these limitations, strong trends further suggest that in addition to the central area, two concentric areas are developed outwards. These results suggest a pre-programmed cartilage growth pattern. If the donor was removed from the analyses, statistical significances were detected, despite being made only on the remaining five donors. On the other hand, the opposite growth pattern found does demonstrate that an exception to the proposed pre-programming is possible. Yet, the donor had a patella fracture and a posterior cruciate ligament rupture around at the age of 30 years, and as pMC levels across the medial condyle are very similar, only minor changes in pMC levels would change the order of growth. Finally, it is worth noticing that the growth on the lateral condyle (i.e. with less weight baring) is similar to the other donors, further suggesting the influence of changed mechanics due to the trauma.

Other comparable human studies do not exist. Instead, the growth has been examined in mice by tracing cell-lineages. The central/middle area on the tibia condyles had the largest expansion of progenitors [118]. Thus, the central area seems to be the initial location of development. Further, the cells in the superficial zone have been shown to have proliferating properties, possibly being responsible for the lateral expansion of the cartilage matrix [39,40,118–121]. In the current setup, the samples analysed were full-thickness and therefore zonal information is unavailable.

4.1.3 Timing

The estimated donor age at tissue formation was calculated using the measured pMC level and the donor birth year (section 3.5.1) resulting in a mean age of 11.7 years (±3.8 SD) with almost every sample ranging between 5–20 years (Fig. 5A). From the samples at similar locations on each of the condyles, the age at formation medially was calculated to 11.4 years (±1.2 SD) and 10.3 years (±1.1 SD) laterally with a significant difference found (P = 0.009) between the two condyles (Fig. 5B).

The average age at formation across the entire tibia plateau was 7–16 years as shown schematically (Fig. 5C).

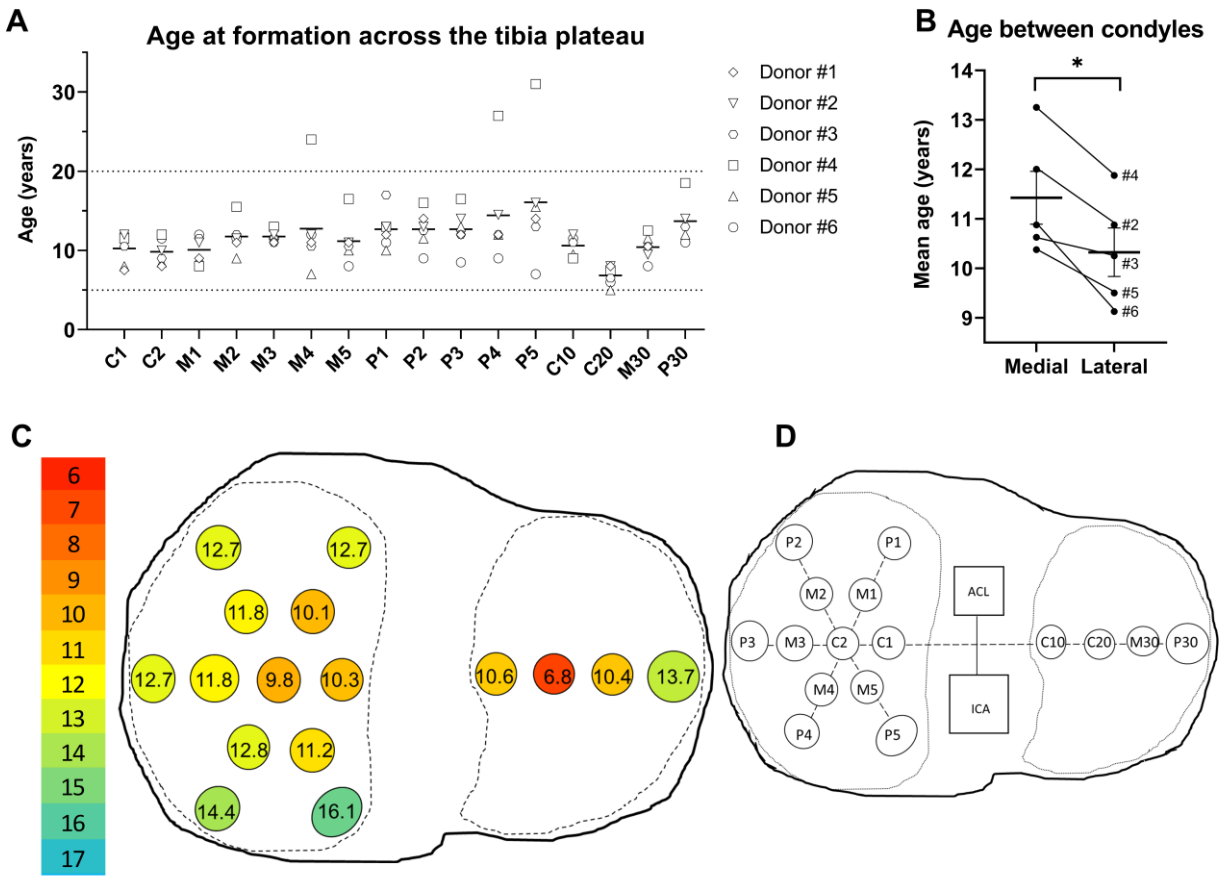


Figure 5: Timing of cartilage growth

A: Corresponding age at formation at every location. Values are individual data points in years, with lines representing the area means. **B:** The age at formation between each condyle. Values are means in years of four samples on each condyle for each donor (n=5 due to missing values in donor #1) and error bars represent condyle means ±SEM. **C:** Schematic presentation of the age at formation on the tibia plateau. Values are mean years. **D:** Location legend. ACL: Anterior cruciate ligament. ICA: Inter condylar area. * equals $P < 0.05$

The data on trypsin-resistant matrix of cartilage collagen contained ^{14}C levels equivalent to levels in the atmosphere at donor age of 7–16 years, a time with rapid peri-pubertal height growth of the skeleton [122]. From the data, there were no samples equivalent to an age below five years and combined with the range, this then suggests either that the matrix is completely renewed, or that the majority is developed in addition to the early childhood collagen. If early childhood collagen remained, new collagen would result in age of formations years after birth, similar to the current

data. Thus, both possibilities exist. At the other end of the range, it seems that most of the collagen mass then remains in adult life without turnover (inert). However, as the pMC levels are a mass-weighted average, smaller amounts of newer incorporated matrix could still be added on to this bulk of "the old or original" matrix, and as shown in figure 5A, some samples did contain pMC levels corresponding to a formation age around 30 years. Thus, any minor collagen incorporation in the trypsin-resistant matrix before and after this range cannot be excluded. Further, cartilage could be maintained by other parts of the matrix not analysed in the current setup, i.e. any new unincorporated collagen or collagen cross-links.

The ^{14}C measurements show that the medial condyle was made around one year later than the lateral condyle. This could be because the medial condyle is bigger and thereby taking longer time to complete. On the other hand, the medial condyle is loaded more [123], and loading could cause this difference in turnover, although as described previously (section 1.4), loading predominately affects GAGs. In children and adolescents, an effect of loading on collagen is unknown, and the consequences of loading in this plastic age-range are unclear. In a cross-sectional setup on school children, the amount of cartilage determined by MRI scans was associated with the time spent being active [124], yet, when measuring longitudinally this association became insignificant [125]. From these sparse combined data, any effect on collagen or even on cartilage itself by exercise in childhood remains unclear.

A low number of donors with different clinical conditions limits this study, and additional donors of preferably healthy cartilage tissue would have enhanced the strength of the growth pattern results. On the other hand, despite the clinical differences later in life, the results were relatively similar. In summary, study I on the life-long incorporation of ^{14}C into the trypsin-resistant matrix showed that during school years, the tibia condyles develop from a central area and radially outwards with the medial condyle finishing approximately one year later than the lateral. Further, very limited collagen incorporation was generally found despite clinical differences in adulthood. These findings presented led to new questions: Are the regional differences actually due to the growth pattern suggested, or could it in fact be due to regional differences in mechanical load?

And whether the cartilage in the late-stage OA is in fact without any turnover, necessitates additional exploration.

4.2 Study II: The regional turnover of late-stage OA cartilage matrix

Study II is a prospective cohort study using deuterium labelled alanine (D-ala) incorporation to determine the regional turnover on late-stage OA cartilage.

4.2.1 Participant characteristics

During March 2018 to October 2019, patients were recruited for participation with a total of 20 participants completing the intervention yielding 21 tibia plateaus for analyses (Fig. 6 and table 3) as one participant had bilateral surgery. After the initial bolus only, minor short-lasting dizziness was described by three participants.

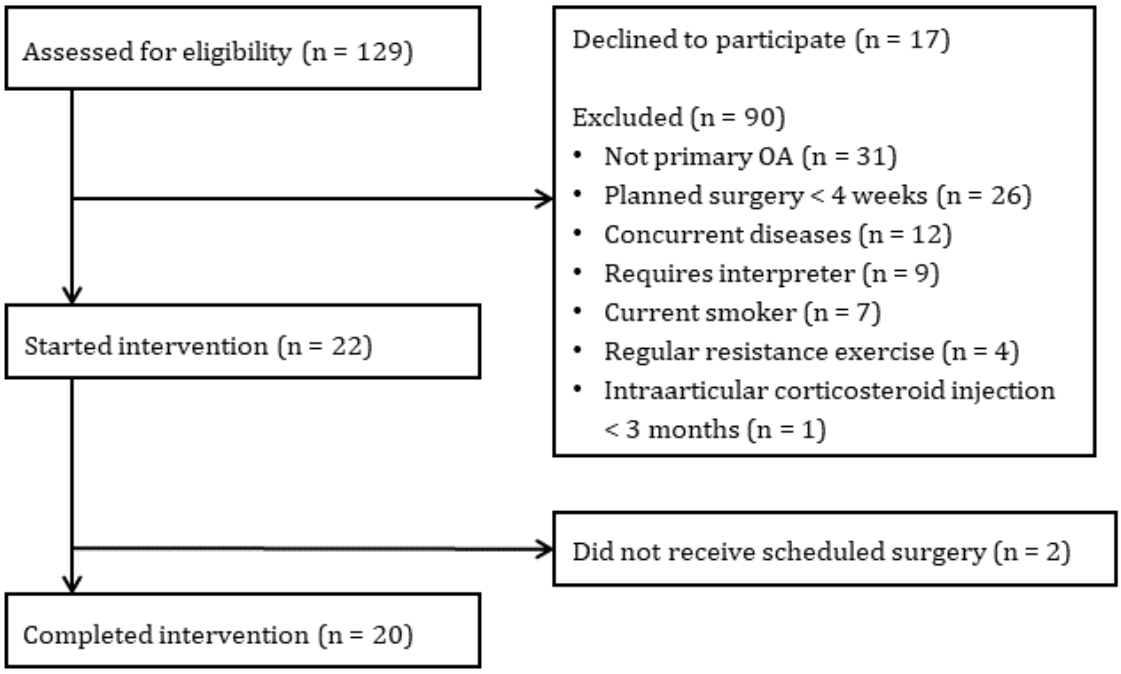


Figure 6: CONSORT flow chart.
The flow of participants through the current study (study II).

Table 3: Participants' characteristics.

Parameter	Value
Age (years)	64.5 ± 9.1
Body mass index (kg/m^2)	29.5 ± 4.8
Sex (number of female:male)	9:11 (45% / 55%)
Kellgren-Lawrence grade (0-4)	3.5 ± 0.5
Labelling duration (days)	28.1 ± 1.1
Daily medication intake (numbers of participants)	
Analgesics	12 (60%)
Acetaminophen/paracetamol	10
NSAID (e.g. ibuprofen)	8
Opioids (e.g. tramadol and morphine)	2
Anti-thrombotic	4 (20%)
Anti-hypertensive	8 (40%)
Anti-hyperlipidaemic	4 (20%)

NSAID = non-steroid anti-inflammatory drug.
Values are means ± SD or the number of participants and the percentage.

4.2.2 Regional cartilage turnover

In this study, blood samples were taken to measure the D-ala enrichment. The blood sample at two hours after the initial ingestion was chosen as the expected time-point of maximum body water enrichment [113]. The plasma precursor enrichment of free D-ala showed the highest median enrichment of 0.65% atomic percent excess (APE) with interquartile range of [0.55–0.78] two hours following the initial 150 ml bolus. Two weeks prior to surgery (the time-point expected to contain the lowest precursor enrichment), the median enrichment was 0.40 [0.29-0.45] %APE. Thus, similar to others[111], an assumption of a relatively stable range of plasma enrichment during the labelling period can be made, albeit without similar level of accuracy.

As a positive control of oral ingestion of heavy water leading to D-ala incorporation into proteins, the plasma protein D-ala enrichment at time of surgery was used. This showed an enrichment of 0.18 [0.17–0.20] %APE (Fig. 7A), thus confirming the protein incorporation. In the current study, median D-ala in cartilage tissues were similar in central 0.063 [0.062-0.063] %APE and submeniscal regions 0.063 [0.062-0.063] %APE, but higher in osteophytes at 0.072

[0.066-0.085] %APE (Fig. 7B). When the regions were compared statistically, post hoc tests resulted in significance ($P < 0.001$) between osteophyte compared to both central and submeniscal regions (Fig. 7B).

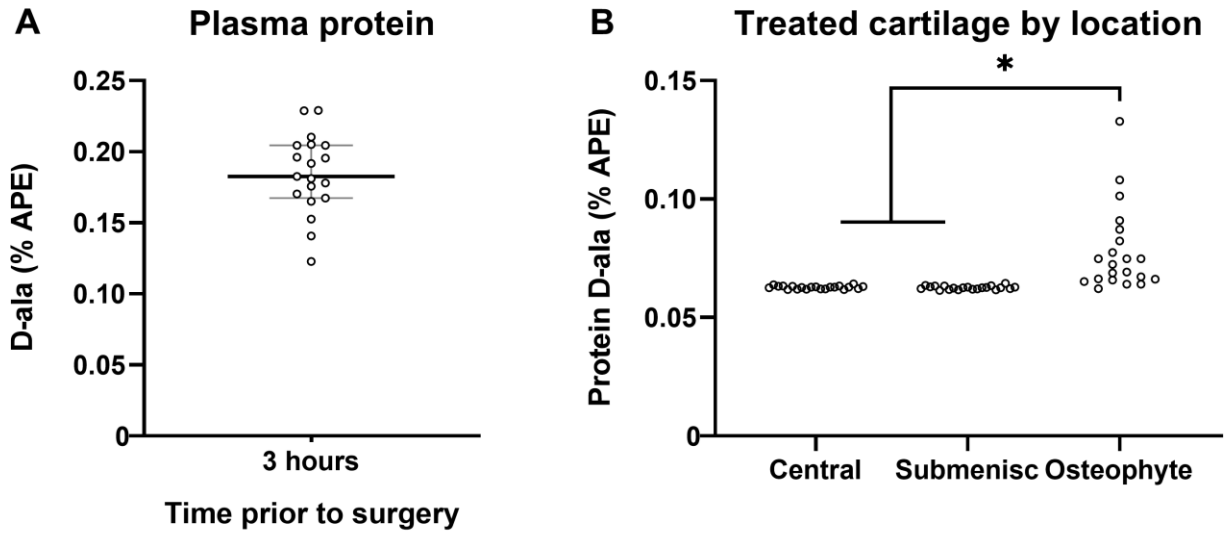


Figure 7: Protein turnover

A: D-ala levels in plasma proteins at the day of surgery. **B:** Regional D-ala in treated cartilage tissue from different locations. Values are individual data points and error bars represent median with interquartile range. D-ala: ^{2}H-alanine. APE: atomic percentage excess. * equals $P < 0.05$

Osteophytes are bony outgrowths covered by fibro-cartilage [126]. The minor but significant increase in deuterium incorporation compared to proper articular cartilage found in study II is somewhat unexpected, as osteophytes can develop quite rapidly, especially in murine models of OA [127]. As this is the first study to measure the covering cartilage's turnover, no comparisons to the literature can be made. Osteophyte growth is primarily due to the underlying bone, and therefore, the cartilage on osteophytes is possibly made continuously for supporting the bony growth by deep zone ossification, while keeping the top-lining cartilage more or less stable. This top-lining cartilage would be uncalcified and thus sampled for analysis in the current setup.

Gene expression analysis led to sufficient detection of four genes in addition to GAPDH (Fig. 8). There was no difference in GAPDH, thus validating the use of RPLPo for normalisation. When compared to central region, submeniscal region had significantly increased MMP-3 by 4.1–fold ($P < 0.0001$) and decreased lubricin expression by 0.6–fold ($P = 0.0443$) (Fig. 8).

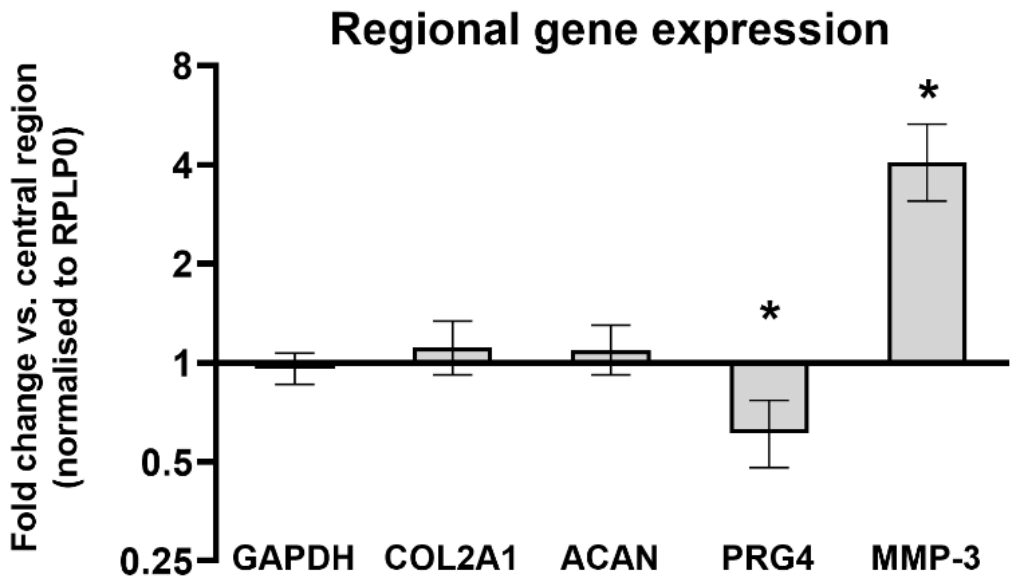


Figure 8: Cartilage mRNA gene expression in submeniscal region
The gene expression of sufficiently detected targets relative to central location normalised to RPLP0. Values are geometric means ± SEM (back transformed) shown on a logarithmic scale. * equals $P < 0.05$ compared to central location

The D-ala results on proper articular cartilage determine that the regional turnover is similar. This directly adds to the results of study I, which suggested a growth pattern based on life-long measurements of ^{14}C incorporation, however, these results could also have been due to regional differences in turnover because of unequal loading [128]. Now, by the direct measurement of later-in-life deuterium-labelled alanine incorporation, our current data confirm a similar turnover across the medial condyle despite unequal loading. However, similarity in collagen expression combined with the difference in gene expression with increased MMP-3 submeniscally, does mean that despite the upregulation of MMP-3, this is not able to change the (collagen) turnover under the meniscus. Thus, either the MMP-3 is not translated into more active MMP-3 protein, or the breakdown of the trypsin-resistant matrix (collagen) is not influenced by MMP-3. Currently, neither measurements of active MMP-3 enzymes nor assessment of the MMP-3 cleaving capacity on this particular matrix were made, and thus remains inconclusive.

The low levels of trypsin-resistant cartilage D-ala compared to plasma protein D-ala do imply a low capacity for synthesis into the mainly stabile collagen in OA cartilage within the last four

weeks prior to sampling. As described in section 1.2, collagen formation assessed by isotope incorporation in OA cartilage was found to be very limited in one [30] but increased in another study[32], both examining a similar enzymatically treated matrix fraction as the current setup. The increased incorporation of tritium (^3H) was examined in five dogs, eight days after intra-articular injections (only one had intravenous injection instead) and conducted in the weeks after transection of the anterior cruciate ligament [32]. This early period following surgery is at best representative of post-traumatic OA, but more likely, it represents an acute traumatic response, both of which are different from the current human data on late-stage primary OA, and the results are thus incomparable. In study I, a donor had few samples from an area that contained pMC levels corresponding to incorporation around the age of 30 years. Nonetheless, regardless of the regional location, the current data on 21 plateaus compared to plasma proteins could suggest that the trypsin-resistant matrix has limited incorporation of any newly synthesised proteins. The current study was constructed to detect an incorporation of deuterium over the longest logistical period possible, prioritising minimum hospital visits and tissue collections in order to recruit a sufficient number of participants within a feasible time frame. By doing so, the fractional synthesis rate (FSR) cannot be accurately calculated as the precursor enrichment necessary is unclear, and thus an ability to specify an amount of renewability remains impossible.

4.2.3 Untreated cartilage turnover

In untreated and enzymatically treated parts of the same cartilage samples from central (n=2), submeniscal (n=2), and osteophytes (n=6), the median D-ala were 0.075 [0.071-0.078] % APE in untreated and 0.065 [0.062-0.070] % APE in treated samples (Fig. 9). This was consistently in every sample, and paired analysis showed a statistically significant difference ($P < 0.0001$).

Enzymatic treatment was used to isolate the stabile collagen fraction of cartilage. The removed matrix is largely composed of proteoglycans (aggrecan in particular) and GAGs, but also other matrix molecules (section 1.1.). In a subset of samples being of sufficient size, a part was left untreated for D-ala measurements, and the difference between treated and untreated (raw) cartilage demonstrates that untreated cartilage has a higher turnover. Others have examined the

turnover in raw cartilage by continuous infusion of stabile isotopes ($^{13}C_6$-phenylalanine) in the hours prior to knee replacement surgery due to OA, showing an FSR ofo.96%/day [129]. The cartilage sampled was healthy looking (thus suspected to be submeniscal), and when compared to muscle tissue from the same individual, similar turnover kinetics were found [129]. This is a rather high FSR, however, the synthesis of aggrecan increases with OA (section 1.2.1). Further, by measuring the accumulation of pentosidine (a fluorescent AGE), aggrecan was found to be "younger" in OA [11]. However, whether this was due to increased synthesis de novo, or removal of older, broken down fragments (section 1.2.1) from the matrix could not be determined, yet, the high FSR in raw cartilage reported [129] could very well support synthesis.

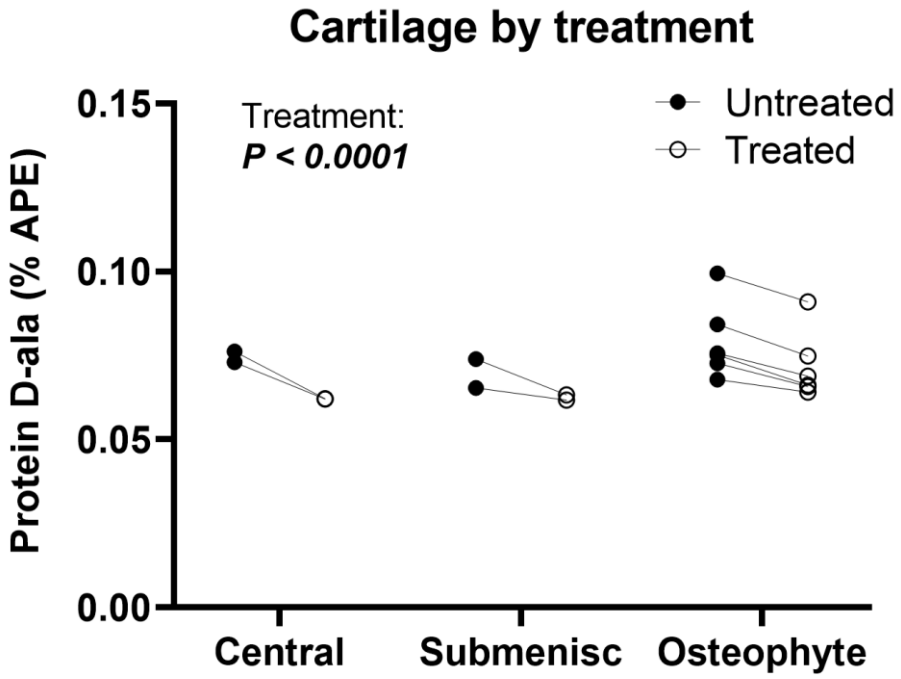


Figure 9: Effect of cartilage treatment
Cartilage samples were measured regionally in an untreated and treated part.
D-ala: ^{2}H-alanine. APE: atomic percentage excess.

The major limitation of the setup is the lack of specificity, i.e. which specific component (protein) is incorporated with deuterium labelled alanine. However, study I demonstrated that the trypsin-resistant matrix (identical to which the current turnover measurements were made) contained approximately 70% collagen and 1% GAGs. Regardless of the specific protein, this does mean that

apart from fibrillary collagen, other structures in the trypsin-resistant matrix are similarly without any major turnover.

In summary, the data show that despite regional differences in gene expression, the turnover of the articular cartilage matrix across the entire joint surface is similar, and higher in osteophyte cartilage. Further, in late-stage OA, cartilage has a fraction of matrix proteins with a higher turnover compared to the trypsin-resistant matrix of predominately fibrillary collagen.

4.3 Study III: The regional effect of exercise

Study III is an RCT using gene expression to characterise the regional effect of acute loading (using resistance exercise) on late-stage OA cartilage.

4.3.1 Participant characteristics

A total of 32 participants were recruited between May 2018 and September 2019 with a single drop-out (Fig. 10) leading to comparable characteristics in the two groups (table 4). The exercise intervention did not result in any harms reported.

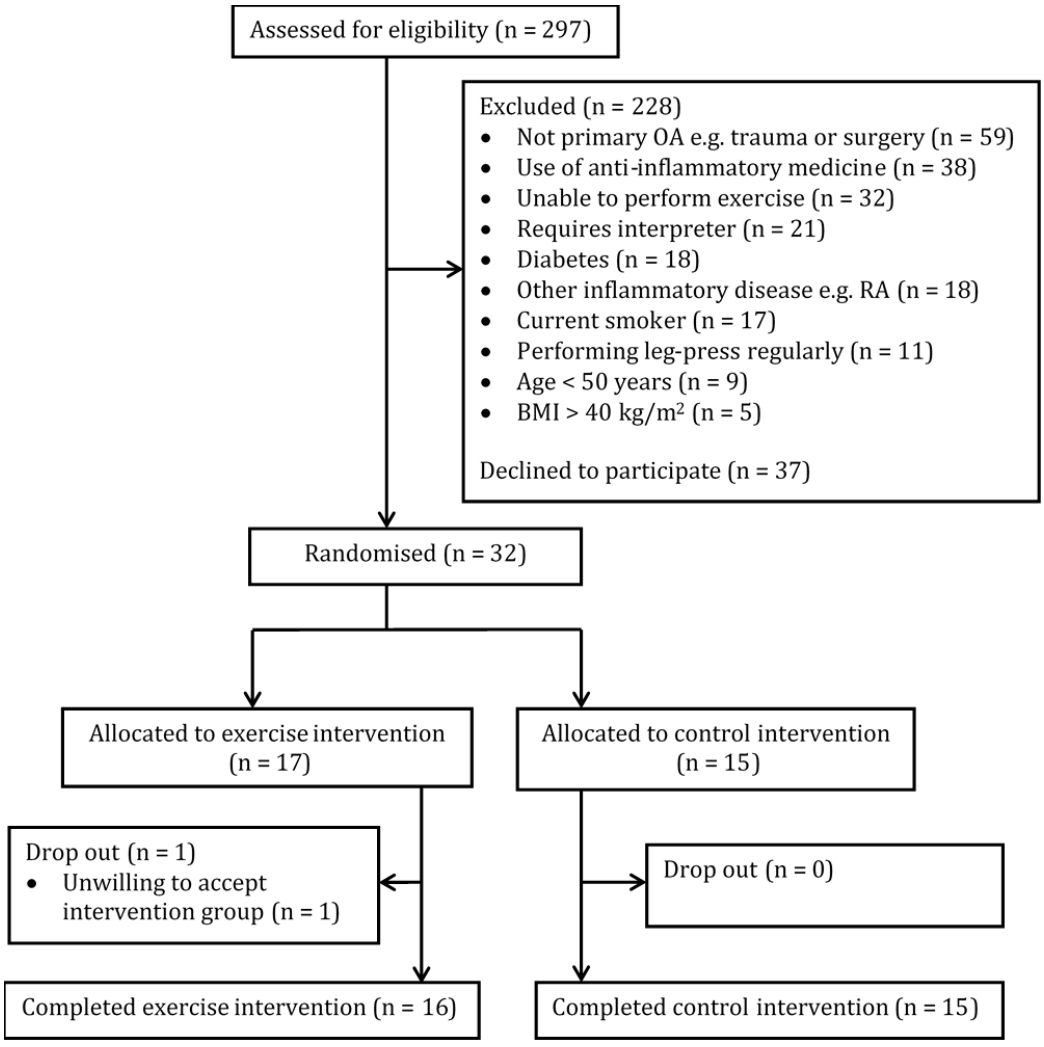


Figure 10: CONSORT flow chart.
The flow of patients/participants through the current study from May 2018 and September 2019. RA: Rheumatoid arthritis.

Table 4: Participants' characteristics.

Parameter	Control (n = 15)	Exercise (n = 16)
Age (years)	67.8 ± 8.0	69.5 ± 7.2
Body mass index (kg/m2)	29.7 ± 3.2	29.2 ± 5.4
Sex (percentage female)	67%	50%
Kellgren-Lawrence grade (0-4)	3.6 ± 0.5	3.4 ± 0.5
5 repetition maximum (kg)	N/A	112.5 ± 31.9
Time from exercise to tissue freezing (h:mm)	N/A	3:24 ± 0:37

N/A: Not applicable. Values are mean ± SD

4.3.2 The effect of exercise

Of the 23 genes that were measured, 13 genes were adequately detected for analysis having more than 10 copies detected (Fig. 11 and table S1). The exercise intervention influenced tissue growth factor beta 1 (TGF-β1) with a significant 1.23-fold increase (P = 0.048), while no other target showed significant change (Fig. 11B).

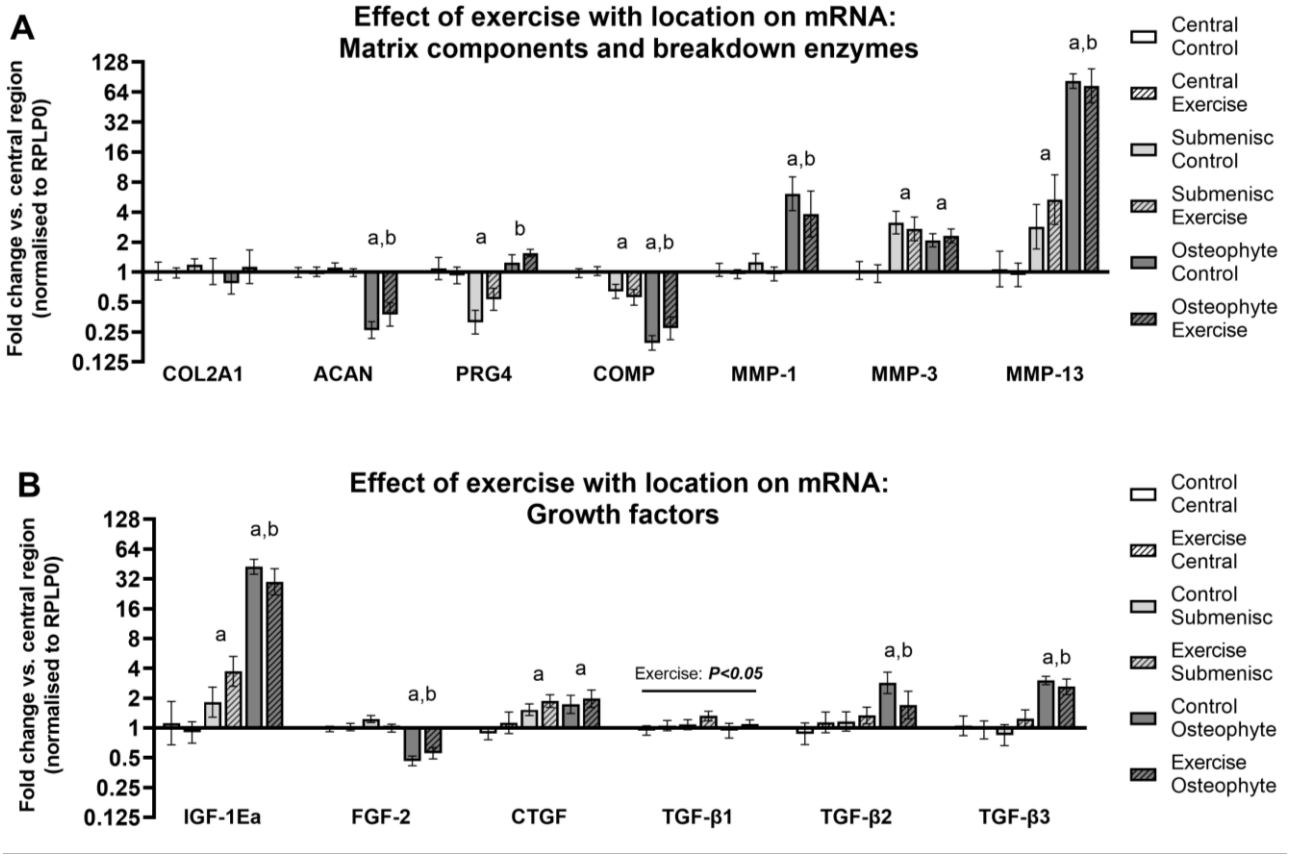


Figure 11: The effect of exercise on gene expression.
The fold change of every region is shown for sufficiently detected targets relative to central region normalised to RPLPo. **A:** Matrix components and breakdown enzymes. **B:** Growth factors. Regions are shown in white (central), light grey (submenisc), and dark grey (osteophyte) colours and exercise by striped fill pattern on top. Values are geometric means ± back transformed SEM. **a** and **b** equals P < 0.05 of main effect vs. central or submeniscal location respectively.

The two-way ANOVA used on the current data does not take testing of multiple targets into consideration, and thus, this barely significantly finding of a limited increase in TGF-β1 expression could very well be due to tests on multiple targets. Hence, our result indicates that in OA cartilage from humans, TGF-β1 expression are possibly able to respond to exercise. Nonetheless, increased

TGF-β1 expression has also been found after *in vitro* loading of healthy aged bovine cartilage [130] and human OA synovial fibroblasts [131], where TGF-β1 exerts an anti-inflammatory effect [132].

As the current study is the first to determine the human *in vivo* effect of exercise, direct comparisons with the literature are unavailable. This also means that in human chondrocytes from OA cartilage, gene expression might not be responsive to a single bout of resistance exercise at the measured time point. In the current study, exercise did not change any matrix-influencing or -component gene. From section 1.4, moderate load on healthy cartilage led to increases of lubricin, aggrecan, IL-4, and IL-10, while limiting the amounts of MMP-1, -3, and -13. Regarding lubricin, a single *in vivo* study in rats with a transection of the anterior cruciate ligament showed reduced lubricin expression by forced running [134], however, forced running could be expected to be overloading, and thus incomparable to the present data. Similar to the current data, loading of OA chondrocytes did not change aggrecan expression, contrary to the increase found in healthy chondrocytes [62]. Currently, interleukin expression was universally undetected, which has previously been reported [135]. This is possibly due to differences in tissue preparation, as *in vitro* demonstrated substantial amounts of IL expression when related to *in vivo* [136], however, increasing IL-10 (protein) was found in healthy rat cartilage after exercise [65]. Similarly, other have found no effect of loading on MMP-1 and -3 on human OA chondrocytes *in vitro* [62,63].

As a single bout of exercise seemed unlikely to change the GAG content in the cartilage matrix within the given timeframe, the extractability by hyaluronidase was examined by immunoblotting. Currently, the immune-assay analysis on the extracted fraction are presented in the thesis as preliminary data, as they need further validation by repetition and other experiments. Thus, these date are not included in the attached manuscript of study III. For CS only, preliminary data on the extracted matrix showed an effect of exercise (P = 0.02) and an effect of location (P = 0.01) without significant interaction (Fig. 12). This suggests an increased release of CS by exercise, however, post hoc test only showed a trend for submeniscal location, and thus, this trend needs verification by further experiments before any additional conclusions on exercise can be made.

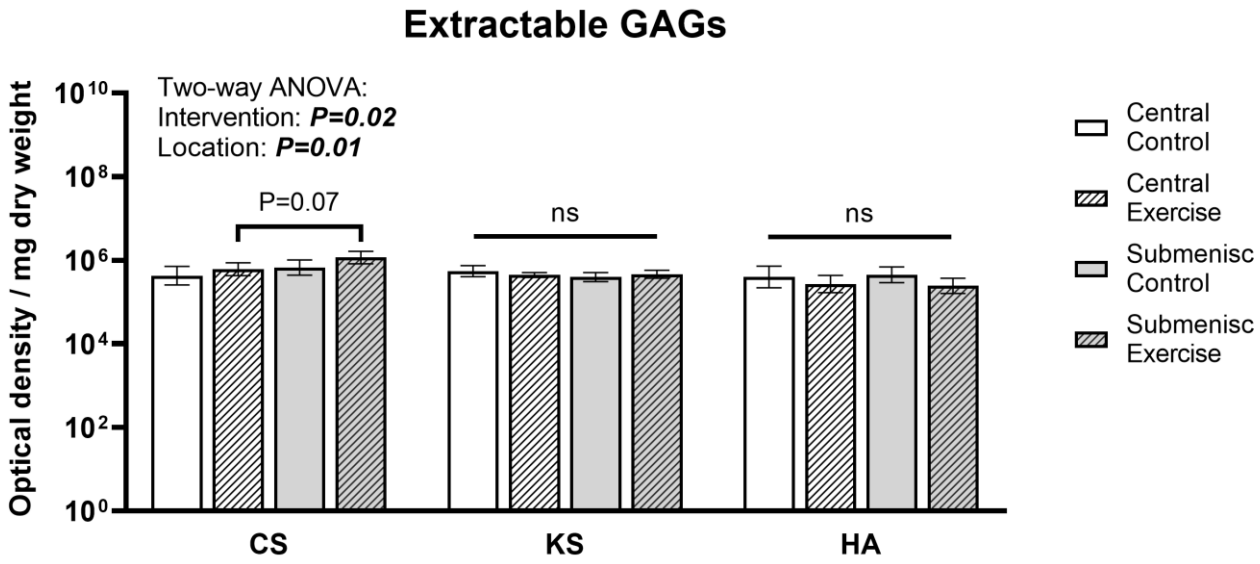


Figure 12: Effect of exercise on glycosaminoglycans
The glycosaminoglycan content extracted by hyaluronidase is shown for articular cartilage regions with the effect of exercise. Values are arbitrary units of optical density presented in geometric means with 95% CI. * equals $P < 0.05$ by intervention. CS: Chondroitin sulphate. KS: Keratan sulphate. HA: Hyaluronic Acid.

As described previously (section 1.5), loading influences cartilage with clinical improvements in OA, and maybe other parts of the trypsin-digestible matrix (the soluble pool of collagen, fibril surface proteins (e.g. COMP), proteoglycans (e.g. aggrecan), lubricin, or interleukins) could in fact be influenced by exercise, and thereby be responsible for the clinical improvements. To shed a bit more light to this matter, the gene expression analysis could possibly be used as a potential pseudo-marker for the trypsin-digestible matrix. In the current setup, the beneficial effect of loading on healthy cartilage could not be verified in OA cartilage. Thus, it seems that an exercise-induced synthesis from chondrocytes are unachievable in OA. The clinical benefits of exercise in OA (section 1.5) are thus suggested to be of extra articular origin, or from synoviocytes. However, whether synoviocytes are able to respond to load by releasing these factors remains unclear, as only few studies are available: A human exercise trial in women with mild to moderate OA found IL-10 in the peri-synovial area [137], and another study applied *in vitro* stretching of synovial fibroblasts and showed increased lubricin gene expression from healthy but not OA donors [131]. Thus, acute exercise has minor if any effect on gene expression under the current setting, and thereby it seems that the beneficial clinical effect of exercise on OA symptoms is most likely due to

extra-articular effects (improved proprioception, joint stability, muscle strength, and possible weight loss).

A limitation in this study is the intervention intensity and timing. It is impossible to determine whether the intervention was a sufficient stimulus given minor effect of the acute load. The exercise protocol did use moderate to hard load to achieve physiological load, but the single exercise bout may have been insufficient. Currently, the gene expression was assessed 3.5 hours following the intervention, and thus, an effect *in vivo* at a later stage cannot be excluded.

4.3.3 Regional differences

On the medial condyle, regional differences in gene expression were found with increased MMP-3, MMP-13, IGF-1, and CTGF, and decreased lubricin and COMP in submeniscal region compared to central region (Fig. 11). A single study has measured the regional gene expression on the human medial tibia condyle [138], and similarly to the current data, they found increased MMP-3, MMP-13, and IGF-1 with decreased COMP expression in undamaged cartilage [138] corresponding to the submeniscial region. No results were reported for CTGF. On the hyaluronidase extracted matrix, a significant increase in the extracted amount of CS under the meniscus compared to central was found (P = 0.01) with no effect on KS or HA (Fig. 12).

The GAG content determined in study I (these data need to be verified in the current setup), showed increased levels of GAGs centrally compared to submeniscal location (see manuscript for study I, figure 2). As aggrecan and COL2A1 expression was regionally similar in the current data and similarly shown by others [139–141], breakdown is possibly the site for regulation. Thus, lower GAG and CS content with increased extractability of CS submeniscally, combined with equal KS extractability, likely results from enzymatic degradation of aggrecan under the meniscus, where CS rich sections are sequentially released while keeping most KS attached (section 1.5). From the results on regional gene expression and GAG dynamics, a careful suggestion could be that matrix breakdown by MMPs, ossification due to IGF-1 and CTGF signalling, and reduced cartilage matrix formation of COMP and lubricin dominates under the meniscus in areas where cartilage does still

exist. Currently, in study II, and by others [138], cartilage fibrillation was universally found centrally, whereas submeniscal cartilage conditions varied from a healthy presence to complete induration down to the underlying bone. Thus, the regional gene expression pattern found, could possibly be leading to the progress of cartilage loss and ultimately complete induration, had the surgery not been performed.

In osteophytes, decreased gene expression of aggrecan, COMP, and FGF-2 and increased MMP-1, MMP-13, TGF-β3, and IGF-1Ea expression were found compared to proper articular cartilage. This is compatible with osteophyte growth and ossification capability. Very limited work has been done in humans on chondrocyte gene expression from osteophytes, but similar increases in TGF-β and unchanged FGF-2 have been found [142], and while IGF-1 was found expressed in osteophytes, the level was not compared to articular cartilage [143]. Thus, osteophyte gene expression differs from articular cartilage, and combined with the results of study II showing increased turnover compared to proper articular cartilage, the resulting pattern reflects osteophyte growth, remodelling, and ossification capability for supporting the underlying bony growth by ossification of deeper cartilage zones while keeping the top-lining uncalcified cartilage more stable.

In summary, the current study set out to determine if exercise influenced gene expression as a pseudo-marker for trypsin-digestible matrix. We did not see an effect of exercise apart from minor increased TGF-β1 expression, and thus, the beneficial response found in healthy cartilage is not reflected in OA within the given timeframe. This suggests that the beneficial effects of exercise are most likely resulting from the known extra-articular musculo-skeletal adaptations. On the other hand, from the lack of effect, exercise appears safe even in late-stage OA. Regional differences in gene expression indicates that an unfavourable environment under the meniscus is present, possibly leading to the bony exposure frequently observed is this region.

5. Conclusions

Throughout this thesis, human knee cartilage from the tibia plateau was treated enzymatically to examine the fraction of mainly stabile/insoluble fibrillary collagen type II. Across the plateau, the cartilage collagen matrix had an estimated year of formation at 11.7 years ranging from 7–16 years, and with central areas on each condyle made earlier in life compared to peripheral areas. In summary, this suggests that a radially cartilage development takes place during skeletal growth when children are in their school years.

The regional turnover (i.e. protein incorporation of deuterium) of cartilage collagen matrix was similar centrally and under the meniscus, and lower compared to plasma proteins, which suggests a restricted renewability capacity of collagen in late-stage OA. Further, catabolic gene expression was found under the meniscus compared to central cartilage and combined with the similar D-ala content (i.e. synthesis *de novo*), matrix degradation, fibrosis, and ossification are suggested to prevail, possibly causing the bony exposure frequently observed in this region. Osteophyte cartilage showed higher turnover and a different gene expression pattern compared to the proper articular cartilage, which reflects the capability for supporting the underlying bony growth by deep zone cartilage ossification while keeping the superficial lining of uncalcified cartilage more stable.

The turnover was higher in untreated compared to the enzymatically treated cartilage, where aggrecan, COMP, lubricin, soluble collagen, and other smaller proteins was removed. Consequently, this fraction could possibly influence cartilage integrity in late-stage OA. The RCT using acute loading intervention only demonstrated a slight increase in gene expression of TGF-β1. Thus, contrarily to the beneficial loading response found in healthy cartilage, in late-stage OA, this was not observed, suggesting that the clinical effects of exercise in OA are most likely resulting from the known extra-articular adaptations.

Taken together, this demonstrates that apart from osteophyte cartilage, the fibrillary collagen type II is initially made centrally and has very limited turnover in adulthood. Further, despite regional differences in gene expression, late-stage OA cartilage collagen turnover is regionally similar and limited, while other matrix components have a higher turnover. Finally, in human late-stage OA, acute loading *in vivo* has minor effects on cartilage.

6. Perspectives

So far, renewability of adult human OA cartilage seems unlikely. However, soluble collagen type II[13] and proteoglycans have been shown to have a faster turnover [11,12,30], and together with collagen cross-links, these could potentially influence cartilage structure, regeneration, and resistance to degradation. Unloading the OA cartilage by knee joint distraction led to reformation of cartilage, although the average GAG content assessed by dGEMRIC was unchanged from baseline, and no details on the collagen matrix was reported [144]. Yet, a correlation between clinical improvement and dGEMRIC was found [144], suggesting that in some patients, GAGs can be detected in the newly formed matrix. A study on patients undergoing tibia osteotomy for knee OA, histologically evaluated the composition of cartilage biopsies sampled arthroscopically at time of surgery and two years later [145]. They showed that GAG content (by safranin-O staining histologically) and cartilage condition (Mankin grading) improved in two thirds of the participants, but rather than demonstrating newly formed hyaline cartilage, the developed tissue was fibrocartilago [145]. Thus, OA cartilage does seem to have the ability to regenerate by forming an unstructured collagen matrix of fibrosis, and possibly new GAGs in some but not all patients. This is compatible with the presented data in this thesis. However, the studies discussed and the thesis' data further demonstrates that the mature composition of hyaline cartilage with optimal collagen structure is effectively unable to regenerate.

This thesis presented a regional difference in expression pattern, suggesting a possible cause of the typically macroscopic appearance of degradation under the meniscus. The catabolic pattern is usually seen with overloading, yet, loading is higher centrally. The meniscus could be the influencing factor, and it might be that the loading type under the meniscus is worse than the one centrally. However, meniscectomies are a great risk factor for OA [146], and thus the meniscus seems not to be the culprit but a great protector. The trigger for OA is mechanical overloading with loss of GAGs superficially leading to collagen damage and catabolic response with breakdown enzymes as a repair attempt (section 1.4 & 1.5). The central cartilage was uniformly observed to be thicker than the submeniscal. Given the growth pattern results in study I, the older cartilage centrally could possibly have grown stronger, as the years of cartilage growth could likely be a plastic period with possible effects on adult cartilage health. The effect of exercise in young healthy cartilage is uncertain, but exercise provides bigger bones and thereby bigger

joints, and thus more cartilage [147], and it could be speculated that more cartilage takes longer time to degenerate and erode away, thus reducing the risk of OA development later in life.

Continuing down the path of the loading effect on healthy cartilage, the regional differences in GAG content (higher centrally), could be due to long-term adaptation to the higher load centrally even after development. This might influence susceptibility for developing primary OA in adulthood. This thesis could not demonstrate a likely protective effect of exercise in late-stage OA, however, in healthy patients with medial meniscus resection 3–5 years earlier, an increase in femoral cartilage was found after four months of regular exercise [148,149]. Thus, a possibility for a protective effect of regular exercise prior to OA seems plausible. It is intriguing to speculate, that healthy cartilage is due to the right amount of load at the right time [150], and regular moderate exercise seems to protect the cartilage due to the loading adaptation similar to the rest of the musculo-skeletal system [151]. One caveat is the sports injury, which has been shown to increase the risk of PTOA several-fold [152].

On the other hand, cartilage and load could be susceptible to the issue of the hen and the egg: Is the loading indeed cartilage protective, or is favourable cartilage (genetics) a requisite for performing high levels of exercise? A study on self-reported symptoms in elite marathon runners actually showed decreased rate of symptoms compared to the population [153], despite the likely overloading. Thus, these runners possibly represent a selected group having the cartilage able to withstand the continued load. Studies examining the cartilage matrix could not show increased cartilage thickness in sprint and weight lifting athletes [154] nor in lifelong active triathletes [155]. However, a larger tibia and patella surface area was found when comparing life-long active triathletes to physically inactive controls doing less than an hour of exercise a week [155]. Thus, the cartilage does indeed seem to be able to adapt to load, albeit not necessarily with increasing thickness as seen in animal models (section 1.4). Apart from PTOA or genetic disorders, OA is rare in individuals below 50 years of age, and thus, the loss of joint function happens after the reproductive period. In evolutionary matters, then cartilage loss would not have provided any pressure for adapting to mechanical usage in contrast to e.g. fractured bones or muscles weakness[5]. Thus, it seems that cartilage is made to last a lifetime, albeit not a lifetime of modern humans. Cartilage is then in the same tissue-category as the eye-lens and teeth; when adequate function is unattainable (i.e. cataract of the lens and damaged or loose teeth) these tissues need to

be replaced. For now, cartilage replacement by joint implants is a valuable treatment for pain and reduced function, and fortunately, much research in this debilitating condition is ongoing. I have great hopes for the future discovery of a disease-postponing treatment to slow the progression to match the current lifetime-expectancy, yet, a disease-modifying treatment or cure seems far away at best. Consequently, prevention possibly by regular moderate loading beginning as early as school age, combined with early treatment of the progressive and irreversible breakdown of fibrillary collagen might be the successful treatment option indeed.

References

1. Dudhia J. Aggrecan, aging and assembly in articular cartilage. *Cell Mol Life Sci*. 2005;62(19-20):2241-2256. doi:10.1007/s00018-005-5217-x

2. Basser PJ, Schneiderman R, Bank R a, Wachtel E, Maroudas A. Mechanical properties of the collagen network in human articular cartilage as measured by osmotic stress technique. *Arch Biochem Biophys*. 1998;351(2):207-219. doi:10.1006/abbi.1997.0507

3. Bank RA, Bayliss MT, Lafeber FP, Maroudas A, Tekoppele JM. Ageing and zonal variation in post-translational modification of collagen in normal human articular cartilage. The age-related increase in non-enzymatic glycation affects biomechanical properties of cartilage. *Biochem J*. 1998;330 (Pt 1:345-351.

4. Benninghoff A. Form und Bau der Gelenkknorpel in ihren Beziehungen zur Funktion. *Zeitschrift für Zellforsch und Mikroskopische Anat*. 1925;2(5):783-862. doi:10.1007/BF00583443

5. Eckstein F, Hudelmaier M, Putz R. The effects of exercise on human articular cartilage. *J Anat*. 2006;208(4):491-512. doi:10.1111/j.1469-7580.2006.00546.x

6. Ludwig TE, Hunter MM, Schmidt TA. Cartilage boundary lubrication synergism is mediated by hyaluronan concentration and PRG4 concentration and structure. *BMC Musculoskelet Disord*. 2015;16(1):386. doi:10.1186/s12891-015-0842-5

7. Waller KA, Zhang LX, Elsaid KA, Fleming BC, Warman ML, Jay GD. Role of lubricin and boundary lubrication in the prevention of chondrocyte apoptosis. *Proc Natl Acad Sci*. 2013;110(15):5852-5857. doi:10.1073/pnas.1219289110

8. Vincent TL, Wann AKT. Mechanoadaptation: articular cartilage through thick and thin. *J Physiol*. 2019;597(5):1271-1281. doi:10.1113/JP275451

9. Heinegård D. Proteoglycans and more--from molecules to biology. *Int J Exp Pathol*. 2009;90(6):575-586. doi:10.1111/j.1365-2613.2009.00695.x

10. Lee H-Y, Han L, Roughley PJ, Grodzinsky AJ, Ortiz C. Age-related nanostructural and nanomechanical changes of individual human cartilage aggrecan monomers and their glycosaminoglycan side chains. *J Struct Biol*. 2013;181(3):264-273. doi:10.1016/j.jsb.2012.12.008

11. Verzijl N, DeGroot J, Bank RA, et al. Age-related accumulation of the advanced glycation endproduct pentosidine in human articular cartilage aggrecan: the use of pentosidine levels as a quantitative measure of protein turnover. *Matrix Biol.* 2001;20(7):409-417.

12. Maroudas A, Bayliss MT, Uchitel-Kaushansky N, Schneiderman R, Gilav E. Aggrecan turnover in human articular cartilage: use of aspartic acid racemization as a marker of molecular age. *Arch Biochem Biophys.* 1998;350(1):61-71. doi:10.1006/abbi.1997.0492

13. Hsueh M-F, Önnerfjord P, Bolognesi MP, Easley ME, Kraus VB. Analysis of "old" proteins unmasks dynamic gradient of cartilage turnover in human limbs. *Sci Adv*. 2019;5(10):eaax3203. doi:10.1126/sciadv.aax3203

14. Struglics A, Hansson M. MMP proteolysis of the human extracellular matrix protein aggrecan is mainly a process of normal turnover. *Biochem J.* 2012;446(2):213-223. doi:10.1042/BJ20120274

15. Vynios DH. Metabolism of Cartilage Proteoglycans in Health and Disease. *Biomed Res Int*. 2014;2014:452315. doi:10.1155/2014/452315

16. Dudhia J, Davidson CM, Wells TM, Vynios DH, Hardingham TE, Bayliss MT. Age-related changes in the content of the C-terminal region of aggrecan in human articular cartilage. *Biochem J.* 1996;313 (Pt 3:933-940.

17. Mankin HJ, Dorfman H, Lippiello L, Zarins A. Biochemical and metabolic abnormalities in articular cartilage from osteo-arthritic human hips. II. Correlation of morphology with biochemical and metabolic data. *J Bone Joint Surg Am.* 1971;53(3):523-537.

18. Eyre DR. Articular cartilage collagen: an irreplaceable framework? *Eur Cells Mater*. 2006;12:57-63. doi:10.22203/eCM.v012a07

19. Wiberg C, Klatt AR, Wagener R, et al. Complexes of Matrilin-1 and Biglycan or Decorin Connect Collagen VI Microfibrils to Both Collagen II and Aggrecan. *J Biol Chem.* 2003;278(39):37698-37704. doi:10.1074/jbc.M304638200

20. Kannus P. Structure of the tendon connective tissue. *Scand J Med Sci Sport*. 2000;10(6):312-320. doi:10.1034/j.1600-0838.2000.010006312.x

21. Molnar JA, Alpert N, Burke JF, Young VR. Synthesis and degradation rates of collagens in vivo in whole skin of rats, studied with 1802 labelling. *Biochem J.* 1986;240(2):431-435. doi:10.1042/bj2400431

22. Grynpas MD, Gahunia HK, Yuan J, Pritzker KPH, Hartmannt D, Tupy JH. Analysis of collagens solubilized from cartilage of normal and spontaneously osteoarthritic rhesus monkeys. *Osteoarthr Cartil Osteoarthr Res Soc*. 1994;2:227-234.

23. Ballou JE, Thompson RC. Studies of metabolic turnover with tritium as a tracer. V. The predominantly non-dynamic state of body constituents in the rat. *J Biol Chem*. 1956;223(2):795-809.

24. Zhou H, Wang S-P, Herath K, et al. Tracer-based estimates of protein flux in cases of incomplete product renewal: evidence and implications of heterogeneity in collagen turnover. *Am J Physiol Endocrinol Metab*. 2015;309(2):E115-21. doi:10.1152/ajpendo.00435.2014

25. Bechshøft CL, Schjerling P, Bornø A, Holm L. Existence of life-time stable proteins in mature rats-Dating of proteins' age by repeated short-term exposure to labeled amino acids throughout age. *PLoS One*. 2017;12(9). doi:10.1371/JOURNAL.PONE.0185605

26. Verzijl N, DeGroot J, Thorpe SR, et al. Effect of collagen turnover on the accumulation of advanced glycation end products. *J Biol Chem*. 2000;275(50):39027-39031. doi:10.1074/jbc.M006700200

27. Maroudas A, Palla G, Gilav E. Racemization of aspartic acid in human articular cartilage. *Connect Tissue Res*. 1992;28(3):161-169.

28. Canoira L, García-Martínez M-J, Llamas JF, Ortíz JE, Torres T De. Kinetics of amino acid racemization (epimerization) in the dentine of fossil and modern bear teeth. *Int J Chem Kinet*. 2003;35(11):576-591. doi:10.1002/kin.10153

29. Stabler T V, Byers SS, Zura RD, Kraus VB. Amino acid racemization reveals differential protein turnover in osteoarthritic articular and meniscal cartilages. *Arthritis Res Ther*. 2009;11(2):R34. doi:10.1186/ar2639

30. Heinemeier KM, Schjerling P, Heinemeier J, et al. Radiocarbon dating reveals minimal collagen turnover in both healthy and osteoarthritic human cartilage. *Sci Transl Med*. 2016;8(346):346ra90. doi:10.1126/scitranslmed.aad8335

31. Lippiello L, Hall D, Mankin H. Collagen synthesis in normal and osteoarthritic human cartilage. *J Clin Invest*. 1977;59(4):593-600. doi:10.1172/JCI108676

32. Eyre DR, Mcdevitt CA, Billinghamii MEJ, Muiri H. Biosynthesis of Collagen and other Matrix

Proteins by Articular Cartilage in Experimental Osteoarthrosis. *Biochem J*. 1980;188:823-837.

33. Hermansson M, Sawaji Y, Bolton M, et al. Proteomic Analysis of Articular Cartilage Shows Increased Type II Collagen Synthesis in Osteoarthritis and Expression of Inhibin βA (Activin A), a Regulatory Molecule for Chondrocytes. *J Biol Chem*. 2004;279(42):43514-43521. doi:10.1074/jbc.M407041200

34. Nelson F, Dahlberg L, Laverty S, et al. Evidence for altered synthesis of type II collagen in patients with osteoarthritis. *J Clin Invest*. 1998;102(12):2115-2125. doi:10.1172/JCI4853

35. Lorenzo P, Bayliss MT, Heinegård D. Altered patterns and synthesis of extracellular matrix macromolecules in early osteoarthritis. *Matrix Biol*. 2004;23(6):381-391. doi:10.1016/j.matbio.2004.07.007

36. Vos PAJM, Mastbergen SC, Huisman AM, et al. In end stage osteoarthritis, cartilage tissue pentosidine levels are inversely related to parameters of cartilage damage. *Osteoarthritis Cartilage*. 2012;20(3):233-240. doi:10.1016/j.joca.2011.12.007

37. Kronenberg HM. Developmental regulation of the growth plate. *Nature*. 2003;423(6937):332-336. doi:10.1038/nature01657

38. Decker RS, Um H-B, Dyment NA, et al. Cell origin, volume and arrangement are drivers of articular cartilage formation, morphogenesis and response to injury in mouse limbs. *Dev Biol*. 2017;426(1):56-68. doi:10.1016/j.ydbio.2017.04.006

39. Hunziker EB, Kapfinger E, Geiss J. The structural architecture of adult mammalian articular cartilage evolves by a synchronized process of tissue resorption and neoformation during postnatal development. *Osteoarthr Cartil*. 2007;15(4):403-413. doi:10.1016/j.joca.2006.09.010

40. Hayes AJ, MacPherson S, Morrison H, Dowthwaite G, Archer CW. The development of articular cartilage: evidence for an appositional growth mechanism. *Anat Embryol (Berl)*. 2001;203(6):469-479. doi:10.1007/s004290100178

41. Julkunen P, Harjula T, Iivarinen J, et al. Biomechanical, biochemical and structural correlations in immature and mature rabbit articular cartilage. *Osteoarthr Cartil*. 2009;17(12):1628-1638. doi:10.1016/j.joca.2009.07.002

42. Rieppo J, Hyttinen MM, Halmesmaki E, et al. Changes in spatial collagen content and collagen network architecture in porcine articular cartilage during growth and maturation. *Osteoarthr*

Cartil. 2009;17(4):448-455. doi:10.1016/j.joca.2008.09.004

43. Poole CA, Flint MH, Beaumont BW. Chondrons in cartilage: ultrastructural analysis of the pericellular microenvironment in adult human articular cartilages. *J Orthop Res*. 1987;5(4):509-522. doi:10.1002/jor.1100050406

44. Gahunia HK, Pritzker KPH. Effect of exercise on articular cartilage. *Orthop Clin North Am*. 2012;43(2):187-199, v. doi:10.1016/j.ocl.2012.03.001

45. Vincent TL. Targeting mechanotransduction pathways in osteoarthritis: a focus on the pericellular matrix. *Curr Opin Pharmacol*. 2013;13(3):449-454. doi:10.1016/j.coph.2013.01.010

46. Vanwanseele B, Eckstein F, Knecht H, Spaepen A, Stüssi E. Longitudinal analysis of cartilage atrophy in the knees of patients with spinal cord injury. *Arthritis Rheum*. 2003;48(12):3377-3381. doi:10.1002/art.11367

47. Hinterwimmer S, Krammer M, Krötz M, et al. Cartilage atrophy in the knees of patients after seven weeks of partial load bearing. *Arthritis Rheum*. 2004;50(8):2516-2520. doi:10.1002/art.20378

48. Arokoski JP, Jurvelin JS, Väätäinen U, Helminen HJ. Normal and pathological adaptations of articular cartilage to joint loading. *Scand J Med Sci Sports*. 2000;10(4):186-198.

49. Säämänen AM, Tammi M, Jurvelin J, Kiviranta I, Helminen HJ. Proteoglycan alterations following immobilization and remobilization in the articular cartilage of young canine knee (stifle) joint. *J Orthop Res*. 1990;8(6):863-873. doi:10.1002/jor.1100080612

50. Haapala J, Arokoski J, Pirttimäki J, et al. Incomplete Restoration of Immobilization Induced Softening of Young Beagle Knee Articular Cartilage After 50-Week Remobilization. *Int J Sports Med*. 2000;21(1):76-81. doi:10.1055/s-2000-8860

51. Jurvelin J, Kiviranta I, Säämänen AM, Tammi M, Helminen HJ. Partial restoration of immobilization-induced softening of canine articular cartilage after remobilization of the knee (stifle) joint. *J Orthop Res*. 1989;7(3):352-358. doi:10.1002/jor.1100070307

52. Leong DJ, Gu XI, Li Y, et al. Matrix metalloproteinase-3 in articular cartilage is upregulated by joint immobilization and suppressed by passive joint motion. *Matrix Biol*. 2010;29(5):420-426. doi:10.1016/j.matbio.2010.02.004

53. Leong DJ, Li YH, Gu XI, et al. Physiological loading of joints prevents cartilage degradation

through CITED2. *FASEB J*. 2011;25(1):182-191. doi:10.1096/fj.10-164277

54. Wilson W, van Burken C, van Donkelaar C, Buma P, van Rietbergen B, Huiskes R. Causes of mechanically induced collagen damage in articular cartilage. *J Orthop Res*. 2006;24(2):220-228. doi:10.1002/jor.20027

55. Saadat E, Lan H, Majumdar S, Rempel DM, King KB. Long-term cyclical in vivo loading increases cartilage proteoglycan content in a spatially specific manner: an infrared microspectroscopic imaging and polarized light microscopy study. *Arthritis Res Ther*. 2006;8(5):R147. doi:10.1186/ar2040

56. Kiviranta I, Tammi M, Jurvelin J, Säämänen AM, Helminen HJ. Moderate running exercise augments glycosaminoglycans and thickness of articular cartilage in the knee joint of young beagle dogs. *J Orthop Res*. 1988;6(2):188-195. doi:10.1002/jor.1100060205

57. Säämänen AM, Tammi M, Kiviranta I, Jurvelin J, Helminen HJ. Levels of chondroitin-6-sulfate and nonaggregating proteoglycans at articular cartilage contact sites in the knees of young dogs subjected to moderate running exercise. *Arthritis Rheum*. 1989;32(10):1282-1292. doi:10.1002/anr.1780321014

58. Van Ginckel A, Baelde N, Almqvist KF, Roosen P, McNair P, Witvrouw E. Functional adaptation of knee cartilage in asymptomatic female novice runners compared to sedentary controls. A longitudinal analysis using delayed Gadolinium Enhanced Magnetic Resonance Imaging of Cartilage (dGEMRIC). *Osteoarthritis Cartilage*. 2010;18(12):1564-1569. doi:10.1016/j.joca.2010.10.007

59. Cotofana S, Ring-Dimitriou S, Hudelmaier M, et al. Effects of exercise intervention on knee morphology in middle-aged women: a longitudinal analysis using magnetic resonance imaging. *Cells Tissues Organs*. 2010;192(1):64-72. doi:10.1159/000289816

60. Ni G-X, Lei L, Zhou Y-Z, et al. Intensity-dependent effect of treadmill running on lubricin metabolism of rat articular cartilage. *Arthritis Res Ther*. 2012;14(6):R256. doi:10.1186/ar4101

61. Ogawa H, Kozhemyakina E, Hung H-H, Grodzinsky AJ, Lassar AB. Mechanical motion promotes expression of Prg4 in articular cartilage via multiple CREB-dependent, fluid flow shear stress-induced signaling pathways. *Genes Dev*. 2014;28(2):127-139. doi:10.1101/gad.231969.113

62. Millward-Sadler SJ, Wright MO, Davies LW, Nuki G, Salter DM. Mechanotransduction via

integrins and interleukin-4 results in altered aggrecan and matrix metalloproteinase 3 gene expression in normal, but not osteoarthritic, human articular chondrocytes. *Arthritis Rheum*. 2000;43(9):2091-2099. doi:10.1002/1529-0131(200009)43:9<2091::AID-ANR21>3.0.CO;2-C

63. Salter DM, Millward-Sadler SJ, Nuki G, Wright MO. Differential responses of chondrocytes from normal and osteoarthritic human articular cartilage to mechanical stimulation. *Biorheology*. 2002;39:97-108.

64. He Z, Leong DJ, Xu L, et al. CITED2 mediates the cross-talk between mechanical loading and IL-4 to promote chondroprotection. *Ann N Y Acad Sci*. 2019;1442(1):128-137. doi:10.1111/nyas.14021

65. Rojas-Ortega M, Cruz R, Vega-López MA, et al. Exercise modulates the expression of IL-1β and IL-10 in the articular cartilage of normal and osteoarthritis-induced rats. *Pathol - Res Pract*. 2015;211(6):435-443. doi:10.1016/j.prp.2015.01.008

66. Jansen NWD, Roosendaal G, Hooiveld MJJ, et al. Interleukin-10 protects against blood-induced joint damage. *Br J Haematol*. 2008;142(6):953-961. doi:10.1111/j.1365-2141.2008.07278.x

67. Millward-Sadler SJ, Wright MO, Lee H, et al. Integrin-regulated secretion of interleukin 4: A novel pathway of mechanotransduction in human articular chondrocytes. *J Cell Biol*. 1999;145(1):183-189.

68. John T, Müller RD, Oberholzer A, et al. Interleukin-10 modulates pro-apoptotic effects of TNF-α in human articular chondrocytes in vitro. *Cytokine*. 2007;40(3):226-234. doi:10.1016/j.cyto.2007.10.002

69. van Meegeren MER, Roosendaal G, Jansen NWD, et al. IL-4 alone and in combination with IL-10 protects against blood-induced cartilage damage. *Osteoarthritis Cartilage*. 2012;20(7):764-772. doi:10.1016/j.joca.2012.04.002

70. Müller RD, John T, Kohl B, et al. IL-10 overexpression differentially affects cartilage matrix gene expression in response to TNF-α in human articular chondrocytes in vitro. *Cytokine*. 2008;44(3):377-385. doi:10.1016/j.cyto.2008.10.012

71. Vincent TL. Mechanoflammation in osteoarthritis pathogenesis. *Semin Arthritis Rheum*. 2019;49(3):S36-S38. doi:10.1016/j.semarthrit.2019.09.018

72. Brandt KD. Yet more evidence that osteoarthritis is not a cartilage disease. *Ann Rheum Dis*.

2006;65(10):1261-1264. doi:10.1136/ard.2006.058347

73. Nickien M, Heuijerjans A, Ito K, van Donkelaar CC. Comparison between in vitro and in vivo cartilage overloading studies based on a systematic literature review. *J Orthop Res*. 2018;36(8):2076-2086. doi:10.1002/jor.23910

74. Hosseini SM, Veldink MB, Ito K, van Donkelaar CC. Is collagen fiber damage the cause of early softening in articular cartilage? *Osteoarthritis Cartilage*. 2013;21(1):136-143. doi:10.1016/j.joca.2012.09.002

75. Arokoski J, Jurvelin J, Kiviranta I, Tammi M, Helminen HJ. Softening of the lateral condyle articular cartilage in the canine knee joint after long distance (up to 40 km/day) running training lasting one year. *Int J Sports Med*. 1994;15(05):254-260. doi:10.1055/s-2007-1021056

76. Oettmeier R, Arokoski J, Roth AJ, Helminen HJ, Tammi M, Abendroth K. Quantitative study of articular cartilage and subchondral bone remodeling in the knee joint of dogs after strenuous running training. *J Bone Miner Res*. 1992;7 Suppl 2:S419-24. doi:10.1002/jbmr.5650071410

77. Goldring SR. Alterations in periarticular bone and cross talk between subchondral bone and articular cartilage in osteoarthritis. *Ther Adv Musculoskelet Dis*. 2012;4(4):249-258. doi:10.1177/1759720X12437353

78. Goldring MB. Chondrogenesis, chondrocyte differentiation, and articular cartilage metabolism in health and osteoarthritis. *Ther Adv Musculoskelet Dis*. 2012;4(4):269-285. doi:10.1177/1759720X12448454

79. Goldring M, Otero M, Plumb D, et al. Roles of inflammatory and anabolic cytokines in cartilage metabolism: signals and multiple effectors converge upon MMP-13 regulation in osteoarthritis. *Eur Cells Mater*. 2011;21:202-220. doi:10.22203/eCM.v021a16

80. Sandy JD, Chan DD, Trevino RL, Wimmer MA, Plaas A. Human genome-wide expression analysis reorients the study of inflammatory mediators and biomechanics in osteoarthritis. *Osteoarthr Cartil*. 2015;23(11):1939-1945. doi:10.1016/j.joca.2015.03.027

81. Loeser RF, Olex AL, McNulty MA, et al. Microarray analysis reveals age-related differences in gene expression during the development of osteoarthritis in mice. *Arthritis Rheum*. 2012;64(3):705-717. doi:10.1002/art.33388

82. Scanzello CR, Plaas A, Crow MK. Innate immune system activation in osteoarthritis: is

osteoarthritis a chronic wound? *Curr Opin Rheumatol*. 2008;20(5):565-572. doi:10.1097/BOR.0b013e32830aba34

83. Loeser RF. Integrins and chondrocyte-matrix interactions in articular cartilage. *Matrix Biol*. 2014;39:11-16. doi:10.1016/j.matbio.2014.08.007

84. Hwang HS, Park SJ, Cheon EJ, Lee MH, Kim HA. Fibronectin fragment-induced expression of matrix metalloproteinases is mediated by MyD88-dependent TLR-2 signaling pathway in human chondrocytes. *Arthritis Res Ther*. 2015;17(1):320. doi:10.1186/s13075-015-0833-9

85. Kim HA, Cho M-L, Choi HY, et al. The catabolic pathway mediated by Toll-like receptors in human osteoarthritic chondrocytes. *Arthritis Rheum*. 2006;54(7):2152-2163. doi:10.1002/art.21951

86. Orlowsky EW, Kraus VB. The role of innate immunity in osteoarthritis: when our first line of defense goes on the offensive. *J Rheumatol*. 2015;42(3):363-371. doi:10.3899/jrheum.140382

87. Syx D, Tran PB, Miller RE, Malfait AM. Peripheral Mechanisms Contributing to Osteoarthritis Pain. *Curr Rheumatol Rep*. 2018;20(2). doi:10.1007/s11926-018-0716-6

88. Loeser RF, Collins JA, Diekman BO. Ageing and the pathogenesis of osteoarthritis. *Nat Rev Rheumatol*. 2016;12(7):412-420. doi:10.1038/nrrheum.2016.65

89. Bijlsma JWJ, Berenbaum F, Lafeber FPJG. Osteoarthritis: an update with relevance for clinical practice. *Lancet (London, England)*. 2011;377(9783):2115-2126. doi:10.1016/S0140-6736(11)60243-2

90. Altman R, Asch E, Bloch D, et al. Development of criteria for the classification and reporting of osteoarthritis: Classification of osteoarthritis of the knee. *Arthritis Rheum*. 1986;29(8):1039-1049. doi:10.1002/art.1780290816

91. Glyn-Jones S, Palmer AJR, Agricola R, et al. Osteoarthritis. *Lancet*. 2015;386(9991):376-387. doi:10.1016/S0140-6736(14)60802-3

92. Price AJ, Alvand A, Troelsen A, et al. *Hip and Knee Replacement 2 Knee Replacement*. Vol 392.; 2018.

93. Kolasinski SL, Neogi T, Hochberg MC, et al. 2019 American College of Rheumatology/Arthritis Foundation Guideline for the Management of Osteoarthritis of the Hand, Hip, and Knee. *Arthritis Care Res (Hoboken)*. 2020;72(2):149-162. doi:10.1002/acr.24131

94. Fransen M, McConnell S, Harmer AR, Van der Esch M, Simic M, Bennell KL. Exercise for osteoarthritis of the knee. Fransen M, ed. *Cochrane Database Syst Rev*. January 2015. doi:10.1002/14651858.CD004376.pub3

95. Kraus VB, Sprow K, Powell KE, et al. Effects of Physical Activity in Knee and Hip Osteoarthritis: A Systematic Umbrella Review. *Med Sci Sports Exerc*. 2019;51(6):1324-1339. doi:10.1249/MSS.0000000000001944

96. Bricca A, Struglics A, Larsson S, Steultjens M, Juhl CB, Roos EM. Impact of exercise therapy on molecular biomarkers related to cartilage and inflammation in people at risk of, or with established, knee osteoarthritis: a systematic review and meta-analysis of randomized controlled trials. *Arthritis Care Res (Hoboken)*. October 2018. doi:10.1002/acr.23786

97. Koli J, Multanen J, Kujala UM, et al. Effects of Exercise on Patellar Cartilage in Women with Mild Knee Osteoarthritis. *Med Sci Sports Exerc*. 2015;47(9):1767-1774. doi:10.1249/MSS.0000000000000629

98. Multanen J, Nieminen MT, Häkkinen A, et al. Effects of high-impact training on bone and articular cartilage: 12-month randomized controlled quantitative MRI study. *J Bone Miner Res*. 2014;29(1):192-201. doi:10.1002/jbmr.2015

99. Munukka M, Waller B, Rantalainen T, et al. Efficacy of progressive aquatic resistance training for tibiofemoral cartilage in postmenopausal women with mild knee osteoarthritis: a randomised controlled trial. *Osteoarthr Cartil*. 2016;24(10):1708-1717. doi:10.1016/j.joca.2016.05.007

100. Multanen J, Rantalainen T, Kautiainen H, et al. Effect of progressive high-impact exercise on femoral neck structural strength in postmenopausal women with mild knee osteoarthritis: a 12-month RCT. *Osteoporos Int*. 2017;28(4):1323-1333. doi:10.1007/s00198-016-3875-1

101. Saghaei M, Saghaei S. Implementation of an open-source customizable minimization program for allocation of patients to parallel groups in clinical trials. *J Biomed Sci Eng*. 2011;04(11):734-739. doi:10.4236/jbise.2011.411090

102. Schmidt MB, Mow VC, Chun LE, Eyre DR. Effects of proteoglycan extraction on the tensile behavior of articular cartilage. *J Orthop Res*. 1990;8(3):353-363. doi:10.1002/jor.1100080307

103. Rosenblum G, Van den Steen PE, Cohen SR, et al. Direct Visualization of Protease Action on

Collagen Triple Helical Structure. Buehler MJ, ed. *PLoS One*. 2010;5(6):e11043. doi:10.1371/journal.pone.0011043

104. Neuman RE, Logan MA. The determination of hydroxyproline. *J Biol Chem*. 1950;184(1):299-306.

105. Goodsite ME, Rom W, Heinemeier J, et al. High-resolution AMS 14C dating of post-bomb peat archives of atmospheric pollutants. *Radiocarbon*. 2001;43(28):495-515.

106. Lynnerup N, Kjeldsen H, Heegaard S, Jacobsen C, Heinemeier J. Radiocarbon Dating of the Human Eye Lens Crystallines Reveal Proteins without Carbon Turnover throughout Life. Gazit E, ed. *PLoS One*. 2008;3(1):e1529. doi:10.1371/journal.pone.0001529

107. Kueppers LM, Southon J, Baer P, Harte J. Dead wood biomass and turnover time, measured by radiocarbon, along a subalpine elevation gradient. *Oecologia*. 2004;141(4):641-651. doi:10.1007/s00442-004-1689-x

108. Levin I, Kromer B, Hammer S. Atmospheric Δ 14 CO 2 trend in Western European background air from 2000 to 2012. *Tellus B Chem Phys Meteorol*. 2013;65(1):20092. doi:10.3402/tellusb.v65i0.20092

109. Olsen J, Tikhomirov D, Grosen C, Heinemeier J, Klein M. Radiocarbon Analysis on the New AARAMS 1MV Tandetron. *Radiocarbon*. 2017;59(3):905-913. doi:10.1017/RDC.2016.85

110. Stuiver M, Polach HA. Discussion Reporting of 14C Data. *Radiocarbon*. 1977;19(03):355-363. doi:10.1017/S0033822200003672

111. Brook MS, Wilkinson DJ, Mitchell WK, et al. Skeletal muscle hypertrophy adaptations predominate in the early stages of resistance exercise training, matching deuterium oxide-derived measures of muscle protein synthesis and mechanistic target of rapamycin complex 1 signaling. *FASEB J*. 2015;29(11):4485-4496. doi:10.1096/fj.15-273755

112. Brook MS, Wilkinson DJ, Atherton PJ, Smith K. Recent developments in deuterium oxide tracer approaches to measure rates of substrate turnover: implications for protein, lipid, and nucleic acid research. *Curr Opin Clin Nutr Metab Care*. 2017;20(5):375-381. doi:10.1097/MCO.0000000000000392

113. Busch R, Kim Y-K, Neese R a, et al. Measurement of protein turnover rates by heavy water labeling of nonessential amino acids. *Biochim Biophys Acta*. 2006;1760(5):730-744.

doi:10.1016/j.bbagen.2005.12.023

114. Holm L, O'Rourke B, Ebenstein D, et al. Determination of steady-state protein breakdown rate in vivo by the disappearance of protein-bound tracer-labeled amino acids: a method applicable in humans. *Am J Physiol Endocrinol Metab*. 2013;304(8):E895-907. doi:10.1152/ajpendo.00579.2012

115. Holm L, Kjaer M. Measuring protein breakdown rate in individual proteins in vivo. *Curr Opin Clin Nutr Metab Care*. 2010;13(5):526-531. doi:10.1097/MCO.0b013e32833c3c64

116. Wilkinson DJ, Franchi M V, Brook MS, et al. A validation of the application of D(2)O stable isotope tracer techniques for monitoring day-to-day changes in muscle protein subfraction synthesis in humans. *Am J Physiol Endocrinol Metab*. 2014;306(5):E571-9. doi:10.1152/ajpendo.00650.2013

117. Brzycki M. Strength Testing—Predicting a One-Rep Max from Reps-to-Fatigue. *J Phys Educ Recreat Danc*. 1993;64(1):88-90. doi:10.1080/07303084.1993.10606684

118. Kozhemyakina E, Zhang M, Ionescu A, et al. Identification of a Prg4 -Expressing Articular Cartilage Progenitor Cell Population in Mice. *Arthritis Rheumatol*. 2015;67(5):1261-1273. doi:10.1002/art.39030

119. Li L, Newton PT, Bouderlique T, et al. Superficial cells are self-renewing chondrocyte progenitors, which form the articular cartilage in juvenile mice. *FASEB J*. 2017;31(3):1067-1084. doi:10.1096/fj.201600918R

120. Dowthwaite GP, Bishop JC, Redman SN, et al. The surface of articular cartilage contains a progenitor cell population. *J Cell Sci*. 2004;117(Pt 6):889-897. doi:10.1242/jcs.00912

121. Hattori S, Oxford C, Reddi AH. Identification of superficial zone articular chondrocyte stem/progenitor cells. *Biochem Biophys Res Commun*. 2007;358(1):99-103. doi:10.1016/j.bbrc.2007.04.142

122. Mouritsen A, Aksglaede L, Soerensen K, et al. The pubertal transition in 179 healthy Danish children: Associations between pubarche, adrenarche, gonadarche, and body composition. *Eur J Endocrinol*. 2013;168(2):129-136. doi:10.1530/EJE-12-0191

123. Wong M, Carter D. Articular cartilage functional histomorphology and mechanobiology: a research perspective. *Bone*. 2003;33(1):1-13. doi:10.1016/S8756-3282(03)00083-8

124. Jones G, Glisson M, Hynes K, Cicuttini F. Sex and site differences in cartilage development: A possible explanation for variations in knee osteoarthritis in later life. *Arthritis Rheum*. 2000;43(11):2543-2549. doi:10.1002/1529-0131(200011)43:11<2543::AID-ANR23>3.0.CO;2-K

125. Jones G, Ding C, Glisson M, Hynes K, Ma D, Cicuttini F. Knee Articular Cartilage Development in Children: A Longitudinal Study of the Effect of Sex, Growth, Body Composition, and Physical Activity. *Pediatr Res*. 2003;54(2):230-236. doi:10.1203/01.PDR.0000072781.93856.E6

126. van der Kraan PM, van den Berg WB. Osteophytes: relevance and biology. *Osteoarthr Cartil*. 2007;15(3):237-244. doi:10.1016/j.joca.2006.11.006

127. Blaney Davidson EN, Vitters EL, Bennink MB, et al. Inducible chondrocyte-specific overexpression of BMP2 in young mice results in severe aggravation of osteophyte formation in experimental OA without altering cartilage damage. *Ann Rheum Dis*. 2015;74:1257-1264. doi:10.1136/annrheumdis-2013-204528

128. Mononen ME, Tanska P, Isaksson H, Korhonen RK. A Novel Method to Simulate the Progression of Collagen Degeneration of Cartilage in the Knee: Data from the Osteoarthritis Initiative. *Sci Rep*. 2016;6:21415. doi:10.1038/srep21415

129. Smeets JSJ, Horstman AMH, Vles GF, et al. Protein synthesis rates of muscle, tendon, ligament, cartilage, and bone tissue in vivo in humans. Markofski MM, ed. *PLoS One*. 2019;14(11):e0224745. doi:10.1371/journal.pone.0224745

130. Madej W, van Caam A, Blaney Davidson EN, Hannink G, Buma P, van der Kraan PM. Ageing is associated with reduction of mechanically-induced activation of Smad2/3P signaling in articular cartilage. *Osteoarthr Cartil*. 2016;24(1):146-157. doi:10.1016/j.joca.2015.07.018

131. Jamal J, Roebuck MM, Lee S-Y, et al. Modulation of the mechanical responses of synovial fibroblasts by osteoarthritis-associated inflammatory stressors. *Int J Biochem Cell Biol*. 2020;126:105800. doi:10.1016/j.biocel.2020.105800

132. Kuo S-J, Liu S-C, Huang Y-L, et al. TGF-β1 enhances FOXO3 expression in human synovial fibroblasts by inhibiting miR-92a through AMPK and p38 pathways. *Aging (Albany NY)*. 2019;11(12):4075-4089. doi:10.18632/aging.102038

133. Scholtes S, Krämer E, Weisser M, et al. Global chondrocyte gene expression after a single anabolic loading period: Time evolution and re-inducibility of mechano-responses. *J Cell*

Physiol. 2018;233(1):699-711. doi:10.1002/jcp.25933

134. Elsaid KA, Zhang L, Waller K, et al. The impact of forced joint exercise on lubricin biosynthesis from articular cartilage following ACL transection and intra-articular lubricin's effect in exercised joints following ACL transection. *Osteoarthr Cartil*. 2012;20(8):940-948. doi:10.1016/j.joca.2012.04.021

135. Dunn SL, Soul J, Anand S, Schwartz J-M, Boot-Handford RP, Hardingham TE. Gene expression changes in damaged osteoarthritic cartilage identify a signature of non-chondrogenic and mechanical responses. *Osteoarthr Cartil*. 2016;24(8):1431-1440. doi:10.1016/j.joca.2016.03.007

136. Tsuchida AI, Beekhuizen M, 't Hart MC, et al. Cytokine profiles in the joint depend on pathology, but are different between synovial fluid, cartilage tissue and cultured chondrocytes. *Arthritis Res Ther*. 2014;16(5):441. doi:10.1186/s13075-014-0441-0

137. Helmark IC, Mikkelsen UR, Børglum J, et al. Exercise increases interleukin-10 levels both intraarticularly and peri-synovially in patients with knee osteoarthritis: a randomized controlled trial. *Arthritis Res Ther*. 2010;12(4):R126. doi:10.1186/ar3064

138. Snelling S, Rout R, Davidson R, et al. A gene expression study of normal and damaged cartilage in anteromedial gonarthrosis, a phenotype of osteoarthritis. *Osteoarthr Cartil*. 2014;22(2):334-343. doi:10.1016/j.joca.2013.12.009

139. Brew CJ, Clegg PD, Boot-Handford RP, Andrew JG, Hardingham T. Gene expression in human chondrocytes in late osteoarthritis is changed in both fibrillated and intact cartilage without evidence of generalised chondrocyte hypertrophy. *Ann Rheum Dis*. 2010;69(01):234-240. doi:10.1136/ard.2008.097139

140. Yagi R, McBurney D, Laverty D, Weiner S, Horton WE. Intrajoint comparisons of gene expression patterns in human osteoarthritis suggest a change in chondrocyte phenotype. *J Orthop Res*. 2005;23(5):1128-1138. doi:10.1016/j.orthres.2004.12.016

141. Fukui N, Ikeda Y, Ohnuki T, et al. Regional differences in chondrocyte metabolism in osteoarthritis: A detailed analysis by laser capture microdissection. *Arthritis Rheum*. 2008;58(1):154-163. doi:10.1002/art.23175

142. Uchino M, Izumi T, Tominaga T, et al. Growth Factor Expression in the Osteophytes of the Human Femoral Head in Osteoarthritis. *Clin Orthop Relat Res*. 2000;377(377):119-125.

doi:10.1097/00003086-200008000-00017

143. Middleton J, Arnott N, Walsh S, Beresford J. Osteoblasts and osteoclasts in adult human osteophyte tissue express the mRNAs for insulin-like growth factors I and II and the type 1 IGF receptor. *Bone*. 1995;16(3):287-293. doi:10.1016/8756-3282(94)00040-9

144. Besselink NJ, Vincken KL, Bartels LW, et al. Cartilage Quality (dGEMRIC Index) Following Knee Joint Distraction or High Tibial Osteotomy. *Cartilage*. 2020;11(1):19-31. doi:10.1177/1947603518777578

145. Bergenudd H, Johnell O, Redlund-Johnell I, Lohmander LS. The articular cartilage after osteotomy for medial gonarthrosis. *Acta Orthop Scand*. 1992;63(4):413-416. doi:10.3109/17453679209154757

146. McDermott ID, Amis AA. The consequences of meniscectomy. *J Bone Jt Surg - Ser B*. 2006;88(12):1549-1556. doi:10.1302/0301-620X.88B12.18140

147. Antony B, Jones G, Jin X, Ding C. Do early life factors affect the development of knee osteoarthritis in later life: a narrative review. *Arthritis Res Ther*. 2016;18(1):202. doi:10.1186/s13075-016-1104-0

148. Roos EM, Dahlberg L. Positive effects of moderate exercise on glycosaminoglycan content in knee cartilage: A four-month, randomized, controlled trial in patients at risk of osteoarthritis. *Arthritis Rheum*. 2005;52(11):3507-3514. doi:10.1002/art.21415

149. Hawezi ZK, Lammentausta E, Svensson J, Roos EM, Dahlberg LE, Tiderius CJ. Regional dGEMRIC analysis in patients at risk of osteoarthritis provides additional information about activity related changes in cartilage structure. *Acta Radiol*. 2016;57(4):468-474. doi:10.1177/0284185115591237

150. Buckwalter J a. Sports, joint injury, and posttraumatic osteoarthritis. *J Orthop Sports Phys Ther*. 2003;33(10):578-588. doi:10.2519/jospt.2003.33.10.578

151. Alentorn-Geli E, Samuelsson K, Musahl V, Green CL, Bhandari M, Karlsson J. The Association of Recreational and Competitive Running With Hip and Knee Osteoarthritis: A Systematic Review and Meta-analysis. *J Orthop Sports Phys Ther*. 2017;47(6):373-390. doi:10.2519/jospt.2017.7137

152. Poulsen E, Goncalves GH, Bricca A, Roos EM, Thorlund JB, Juhl CB. Knee osteoarthritis risk is increased 4-6 fold after knee injury - a systematic review and meta-analysis. *Br J Sports Med*.

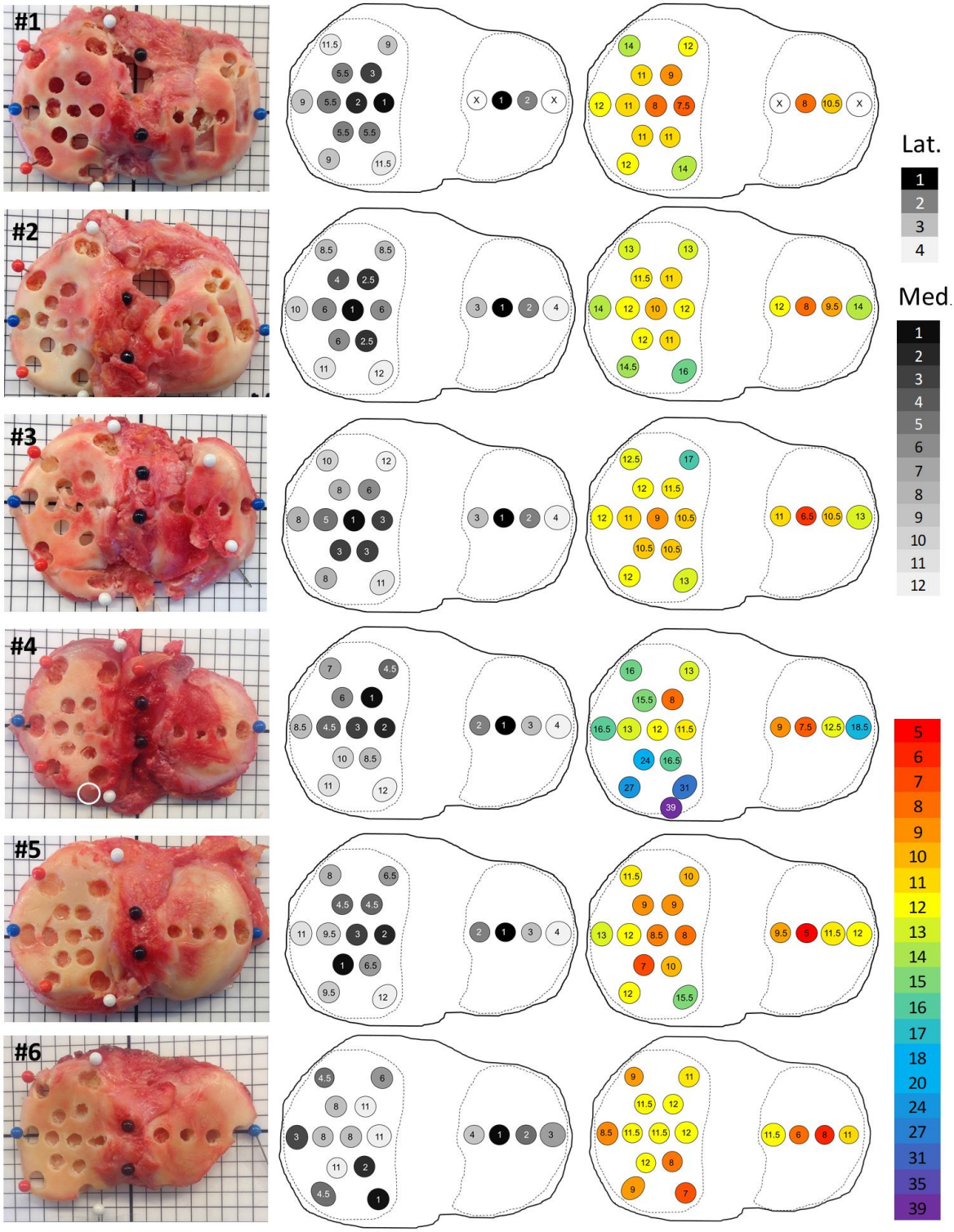
May 2019:bjsports-2018-100022. doi:10.1136/bjsports-2018-100022

153. Ponzio DY, Syed UAM, Purcell K, et al. Low Prevalence of Hip and Knee Arthritis in Active Marathon Runners. *J Bone Jt Surg*. 2018;100(2):131-137. doi:10.2106/JBJS.16.01071

154. Gratzke C, Hudelmaier M, Hitzl W, Glaser C, Eckstein F. Knee cartilage morphologic characteristics and muscle status of professional weight lifters and sprinters: a magnetic resonance imaging study. *Am J Sports Med*. 2007;35(8):1346-1353. doi:10.1177/0363546507299746

155. Eckstein F, Faber S, Mühlbauer R, et al. Functional adaptation of human joints to mechanical stimuli. *Osteoarthritis Cartilage*. 2002;10(1):44-50. doi:10.1053/joca.2001.0480

Figure S1: Details of every plateau used in study I



Left column: Photographs showing donor numbers on the tibia plateau after biopsy sampling. Donors #1–2 supplied healthy cartilage, #3–4 with osteoarthritis (OA), and #5–6 with post-traumatic OA. The plateaus are oriented with anterior direction at the top, and the medial side to the left. From donor #4, an extra biopsy was taken (white circle).
Mid column: Schematic representation of photographs showing the growth pattern from calculated ranks.
Right column: Schematic representation of photographs showing the estimated donor age at formation in years.

Manuscripts

The thesis is based on the following manuscripts:

Study I

Collagen growth pattern in human articular cartilage of the knee.

Adam EM Jørgensen MD, Peter Schjerling PhD, Michael R. Krogsgaard PhD, Michael M. Petersen DMSc, Jesper Olsen PhD, Michael Kjær DMSc & Katja M. Heinemeier PhD

Published in the journal *Cartilage*, doi.org/10.1177/1947603520971016

Study II

The regional turnover of cartilage collagen matrix in late-stage human knee osteoarthritis.

Adam EM Jørgensen MD, Jakob Agergaard PhD, Peter Schjerling PhD & Michael Kjær DMSc

Manuscript not submitted.

Study III

Acute loading has minor influence on human articular cartilage in late-stage knee osteoarthritis: A randomised controlled trial.

Adam EM Jørgensen MD, Peter Schjerling PhD & Michael Kjær DMSc

Manuscript not submitted.

Original Article

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Collagen Growth Pattern in Human Articular Cartilage of the Knee

Adam E.M. Jørgensen[1,2], Peter Schjerling[1,2], Michael R. Krogsgaard[3], Michael M. Petersen[4], Jesper Olsen[5], Michael Kjær[1,2], and Katja M. Heinemeier[1,2]

Abstract

Objective. During skeletal growth, the articular cartilage expands to maintain its cover of bones in joints, however, it is unclear when and how cartilage grows. We aim to determine the expanding growth pattern and timing across the tibia plateau in human knees. *Design*. Six human tibia plateaus (2 healthy, 2 with osteoarthritis, and 2 with posttraumatic osteoarthritis) were used for full-depth cartilage sampling systematically across the joint surface at 12 medial and 4 lateral sites. Methodologically, we took advantage of the performed nuclear bomb tests in the years 1955 to 1963, which increased the atmospheric ^{14}C that was incorporated into human tissues. Cartilage was treated enzymatically to extract collagen, analyzed for ^{14}C content, and year at formation was determined from historical atmospheric ^{14}C concentrations. *Results*. By age-determination, each tibia condyle had central points of formation surrounded by later-formed cartilage toward the periphery. Furthermore, the tibia plateaus contained collagen with ^{14}C levels corresponding to mean donor age of 11.7 years (±3.8 SD). Finally, the medial condyle had lower ^{14}C levels corresponding to formation 1 year later than the lateral condyle ($P = 0.009$). *Conclusions*. Human cartilage on the tibia plateau contains collagen that has experienced little if any turnover since school-age. The cartilage formation develops from 2 condyle centers and radially outward with the medial condyle finishing slightly later than the lateral condyle. This suggests a childhood programmed cartilage formation with a very limited adulthood collagen turnover.

Keywords

articular cartilage, tissue, collagen, tissue, 14C, radiocarbon dating, development

Introduction

Bones in joints are lined with articular cartilage (AC) that allows for near frictionless movement.[1] During childhood and adolescence, bones grow in length and width. The longitudinal growth is well studied: it occurs by cartilage expansion and ossification in the epiphysis and metaphysis until these fully mineralizes into bone, and the skeleton reaches maturity.[2] AC never mineralizes and it is produced by a different pool of chondrocytes; however, the AC growth, and expansion laterally in the joint, during postnatal life is not well understood.[3] Lateral cartilage expansion is essential for maintaining cover of the entire joint surface as growth occurs, and further, a larger surface area of AC would limit the increasing pressure from the rising body weight as the individual grows.

Healthy AC contains around 60 % collagen type II (dry weight).[4] Studies on animals show that apart from the most superficial layer of AC, the initial immature AC is replaced during the skeletal growth prepuberty,[5,6] and during puberty the collagen fibers further develop with a change in orientation and in enzymatic cross-link content into the final mature collagen mesh.[7-9] Similar studies have not been done in humans, but if similar development takes place,

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Supplementary material for this article is available on the *Cartilage* website at https://journals.sagepub.com/home/car.

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Table 1. Donor Characteristics.

Donor	Birth Year	Tissue Type	Tibia Plateau Cartilage Condition
1, male	1971	Healthy cartilage	Normal. No sign of OA.
2, male	1971	Healthy cartilage	Normal. No sign of OA.
3, female	1968	Primary OA	Moderate medial and lateral OA.
4, female	1967	Primary OA	Moderate medial and lateral OA.
5, female	1967	Posttraumatic OA	Moderate medial and lateral OA. Medial meniscus lesion in the twenties (specific age unknown).
6, male	1967	Posttraumatic OA	Moderate medial and lateral OA. Severe patellofemoral OA. Patella fracture and posterior cruciate ligament rupture at age 30 years.

OA = osteoarthritis.

only a minimum of new collagen should be made in adulthood in the mature AC, and the amount of cartilage collagen is determined at some point(s) in time before that. Indeed, human studies show that mature AC has at best a very limited collagen turnover.[10-12] Previous work in our lab has shown limited collagen turnover after the age of approximately 20 years both in healthy and osteoarthritis (OA) affected AC of the tibia plateau.[10] However, as only 2 samples from each donor were analyzed, there are no details available regarding timing and the lateral expanding growth pattern. Thus, we aim to determine whether there are certain time points during growth where a major part of the collagen mesh is laid down, and to reveal the expanding growth pattern across the tibial cartilage in human knees. We hypothesize that the cartilage develops before the age of 20 years along with skeletal maturity, and that central cartilage areas are laid down before peripheral.

Materials and Methods

In the years 1955 to 1963, testing of nuclear bombs led to a dramatic increase in atmospheric ^{14}C levels. After the Test Ban Treaty, a subsequent decline followed, leaving a spike in atmospheric ^{14}C level, known as the "bomb pulse" (**Fig. 3**). Plants incorporate ^{14}C from atmospheric $^{14}CO_2$, which then accumulates in animals and humans that ingest these plants. Tissues with rapid turnover such as muscle have a content of ^{14}C corresponding to the current level in the atmosphere.[13] On the other hand, tissues that are not turned over after the initial formation such as the eye lens[14] will contain ^{14}C levels corresponding to the level present in the atmosphere when the tissue was formed. Therefore, it is possible to determine the turnover of tissues from organisms born in the years spanning the bomb-pulse due to the incorporation of ^{14}C starting from embryogenesis to tissue sampling.

Human Donors

Donors born just after the peak of the bomb pulse were selected, as they would have experienced declining levels of atmospheric ^{14}C since birth. Consequently, a high level of ^{14}C in the collagen matrix would indicate that the matrix is "old," that is, laid down early in life, while a lower level would indicate that the matrix is "young" and therefore laid down later in life. We used 6 whole tibia plateaus for cartilage sampling collected in a previous study[10] and kept at −80 °C (**Table 1**). Two plateaus were collected as waste tissue from patients with healthy cartilage, who had undergone surgery due to primary malignant bone tumors of the distal femur, thus neither affecting the tibia bone nor the cartilage (donors 1 and 2). Four plateaus were from patients with OA: 2 primary and 2 posttraumatic OA (PTOA) collected as waste tissue from total knee replacement surgery (donors 3 to 6). The study was conducted in accordance to the Declaration of Helsinki, and ethical approval for obtaining waste tissue was obtained from the Ethical Committee of the Capital Region of Denmark (H-4-2012-131), and all participants gave written informed consent.

Cartilage Sampling

Using a 5-mm stainless steel punch, 16 cylindrical full depth cartilage samples were taken from each tibia plateau. To standardize sampling, we used a template to obtain 8 biopsies systematically across the entire plateau with additional 8 samples from the larger medial condyle to enhance details in the anterior to posterior direction (**Fig. 1**). Cartilage was separated from the underlying subchondral bone by cutting as close as possible to the bone with a scalpel. Next, the biopsies were cut in thin slices with a scalpel perpendicular to the surface. All biopsy samples were weighed, freeze-dried, and reweighed. A slice of each biopsy was kept as a raw control, that is, without any enzymatic treatment.

Collagen Purification

We aimed at studying the ^{14}C content in collagen, thus we removed glycosaminoglycans (GAGs) and other noncollagenous substances from the remaining slices of the cartilage samples using a collagen extraction procedure as previously

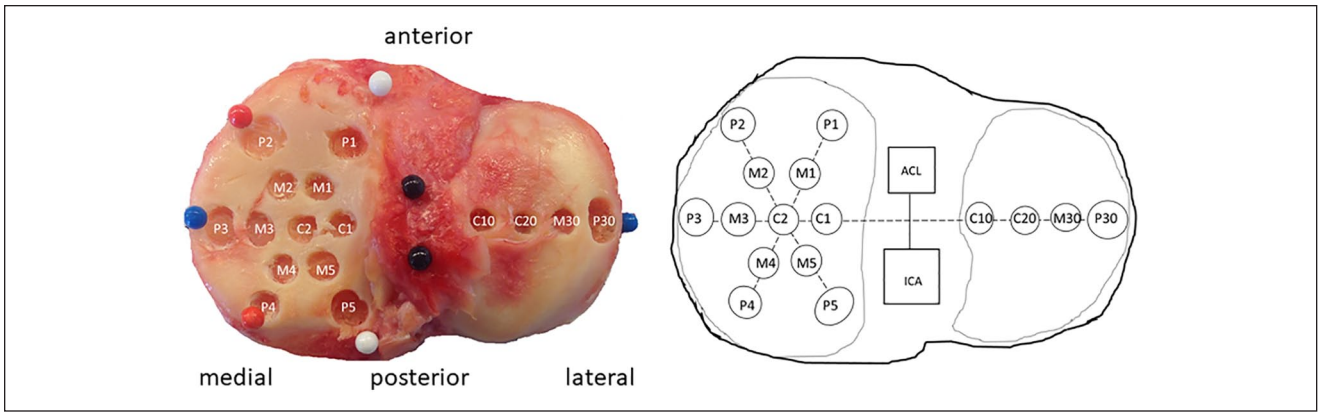

Figure 1. Systematic placement of biopsy locations across the tibia plateau. (**Left**) Photograph of a right tibia plateau after sampling of cartilage tissue. (**Right**) Schematic representation of the same plateau. Biopsies were sampled as follows: The anterior cruciate ligament (ACL) and the intercondylar area (ICA) were located (black pins) marking the anterior-posterior line. A perpendicular line was made giving the longest possible span across (blue pins). Four samples were collected from the most central (C1 and C10) and peripheral (P3 and P30) location possible on each side, and 2 additional samples evenly distributed in between, both medially (C2 and M3) and laterally (C20 and M30). On the medial condyle, we located the points where the cartilage tissue curved from the central to anterior/posterior side near the horns of the medial meniscus (white pins). Two lines between the white pins and sample C2 were drawn and biopsies were taken the most peripheral location possible (P1 and P5) and another evenly between these and C2 (M1 and M5). Two bisecting lines between samples P1-C2 and C2-P3 and between P5-C2 and C2-P3 were marked (red pins). Biopsies were taken the most peripheral location possible (P2 and P4) with another evenly distributed between these and C2 (M2 and M4). This led to systematic sampling of 12 samples medially and 4 laterally.

described.[10] By using enzymatic treatment, the GAGs can be removed with minimal collagen loss.[15] Freeze-dried samples were treated overnight with hyaluronidase (H3506, Sigma) (5 U/mL in 0.05 M sodium acetate and 0.15 M NaCl [pH 6]) at 37 °C. Then, the samples were centrifuged, supernatant removed, and the cartilage slices were washed with isotonic NaCl. Next, trypsin (T8802, Sigma) (1 mg/mL in phosphate buffered saline [PBS]) was added and samples were incubated at 37 °C overnight. To remove any trace of carbon from the added enzymes and the acetate, the samples were washed with PBS, 0.7 M KCl, and 3 times with distilled water before being freeze-dried, weighed, and kept at −80 °C for later analyses. As trypsin is not able to cleave native triple helical collagen,[16] our protocol would leave behind an indigestible cartilage matrix of predominately fibrillar collagen type II, while unincorporated collagen would be removed.

Isotope Analyses

From the enzymatic treated samples, 2 to 3 mg of cartilage slices were sent for isotope analyses (^{13}C, ^{14}C, and ^{15}N) at the AMS ^{14}C Dating Centre, Aarhus University, Denmark. The samples for accelerator mass spectrometry (AMS) were combusted with CuO in sealed combustion tubes at 950 °C and converted to graphite prior to ^{14}C analysis at the 1 MV Tandetron accelerator.[17] The radiocarbon dating results are reported according to international convention[18] and ^{14}C content are given as percent modern carbon (pMC) based on the measured $^{14}C/^{13}C$ ratio corrected for the natural isotopic fractionation by normalizing the result to the standard $\delta^{13}C$ value of −25‰ VPDB (Vienna Pee Dee Belemnite: a $\delta^{13}C$ calibration standard). Stable isotope values of $\delta^{13}C$, $\delta^{15}N$, carbon and nitrogen fraction (by dry weight), and carbon/nitrogen (C/N) atomic ratios were measured at the Aarhus AMS Centre by continuous-flow isotope-ratio mass spectrometry.

Hydroxyproline and Glycosaminoglycan Assay

To verify the extraction of collagen and removal of GAGs, we performed assays detecting hydroxyproline (HYP) and GAGs on all of the remaining enzymatic treated slices and the raw control slice. All were treated with papain (P3125, Sigma) (papain [0.125 mg/mL] in 100 mM sodium phosphate buffer, 10 mM Na_2-EDTA, and 10 mM L-cysteine [pH 6.5]) at 60 °C overnight. For GAG quantification, the diluted papain digest was mixed with 1,9-dimethylmethylene blue (DMMB) (Sigma 341088) solution (38 µM DMMB in 40 mM NaCl, 40 mM glycine, pH 3), and absorbance were read at 595 nm and 540 nm wavelengths (subtracted) and compared with a known standard curve of chondroitin sulphate C (Sigma C4384). For HYP quantification, the papain digest was hydrolyzed in 6 M HCl overnight at 110 °C, dried, rehydrated with distilled water, and dried again before being resuspended in an acetate-citrate buffer (0.6% acetic acid, 130 mM citric acid, 440 mM sodium acetate, 425 mM NaOH, pH 6). A chloramine-T

solution (60 mM chloramine-T, 50% 1-propanol) was added, and samples incubated at room temperature for 20 minutes. Next, an aldehyde perchloric acid solution (1 M 4-dimethylaminobenzaldehyde, 60% 1-propanol, 22% perchloric acid [70%-72%]) was added and incubated for 25 minutes at 60 °C before the reaction was stopped in an ice-bath. The samples were read at 570 nm wavelength and compared with a known standard curve of HYP (Sigma, H1637). As collagen has been found to contain around 13.4% HYP, the concentrations were converted to collagen concentration by multiplying with a factor of 7.5.[19]

Statistics

All statistical analyses were performed using GraphPad Prism version 8.4.1 (GraphPad Software, San Diego, CA, USA). Repeated measures 1-way analysis of variance (ANOVA) or mixed-effects analysis (if values were missing) with the Geisser-Greenhouse correction for sphericity were used. Within areas of each condyle, Holm-Sidak's multiple comparisons test was used *post hoc*.

Friedman test was used for differences in the ranking order of growth with Dunn's multiple comparisons test post hoc within areas on the medial condyle.

Paired *t* test was used for difference between medial and lateral condyles.

Statistical significance was defined as P values <0.05 for all analyses.

Results

Tissue Composition of Raw and Treated Cartilage

The isotope measurements were made on extracted collagen. Thus, to evaluate the effectiveness of the extraction protocol, the content of GAGs and collagen were analyzed for every location. All cartilage samples were freeze-dried initially: The water content in raw cartilage was 70.9% ± 2.7% without any detectable difference between locations (Supplementary Fig. S1A). However, between areas a difference was found ($P = 0.022$), but multiple comparisons test was insignificant (**Fig. 2A**). The extraction protocol successfully reduced the GAG content by 92%: Raw cartilage samples contained 137.3 ± 31.8 µg/mg fry weight (d.w.) GAGs, which decreased to 10.7 ± 2.8 µg/mg d.w. GAGs in treated cartilage without any significant difference between locations (Supplementary Fig. S1B). However, in raw cartilage only, a difference between areas was found ($P = 0.047$), with multiple comparisons test showing difference between M1-M5 and P1-P5 ($P = 0.016$) on the medial condyle (**Fig. 2B**). The extraction protocol expectedly increased the total collagen content: Raw cartilage samples contained 395.9 ± 95.3 µg/mg d.w. collagen, while treated cartilage contained 697.7 ± 111.9 µg/mg d.w. collagen, without any significant difference between locations (Supplementary Fig. S1C). However, in raw cartilage only, a difference between areas was found ($P = 0.038$), but multiple comparisons test was insignificant (**Fig. 2C**). Thus, as expected the treated cartilage samples used for isotope measurements contained high levels of collagen and very low levels of GAGs regardless of location on the plateau.

Growth Pattern

The ^{14}C levels in cartilage collagen from each individual donor at each anatomical location are shown with the historical atmospheric concentrations of ^{14}C for comparison[20,21] (**Fig. 3**), and in details in Supplementary Table S1. To examine differences across the plateau, the growth pattern is presented for each donor at every location (**Fig. 4A**) showing a significant difference across the plateau ($P = 0.049$). As the medial condyle had eight additional samples taken, the means from every area are presented (**Fig. 4B**): A significant test for linear trend was found ($P = 0.007$), and 1-way ANOVA showed a strong trend ($P = 0.056$). To further clarify the growth pattern visually, we used the pMC values (Supplementary Table S1) to rank every sample location from high to low, for each of the condyles: For each donor, the samples were ranked 1 to 12 on the medial condyle, and 1 to 4 on the lateral condyle (with the mean rank used for similar levels), thus 1 being made first/earliest in life and 12 last/latest in life (Supplementary Fig. S2). The median rank for each location is presented (**Fig. 4C**) showing a significant difference across the plateau ($P = 0.015$). We then compared the mean ranks of the areas on the medial condyle, which showed a trend ($P = 0.072$) (**Fig. 4D**). To illustrate the growth pattern on the plateau, a schematic overview of all donors is presented (**Fig. 4E**). Both tibia condyles have central points of formation containing older collagen with younger collagen radially outward from here.

^{14}C Levels and Estimation of Tissue Formation Year

By comparing the ^{14}C levels of each cartilage biopsy with the historical atmospheric ^{14}C levels, a year of tissue formation can be estimated. By relating this year of tissue formation with the birth year of the donor, the age of the donor at the time of cartilage formation can be estimated. For example, for donor 1, tissue in location C1 had a ^{14}C level of 131.54 pMC (Supplementary Table S1), corresponding to the atmospheric level between the years 1978 and 1979 and thus to a donor age of 7.5 years (1978.5-1971). Importantly, this does not necessarily mean that all the tissue in site C1 for this donor was formed at the age of 7.5 years, but merely that the mass weighted average of the ^{14}C levels accumulated

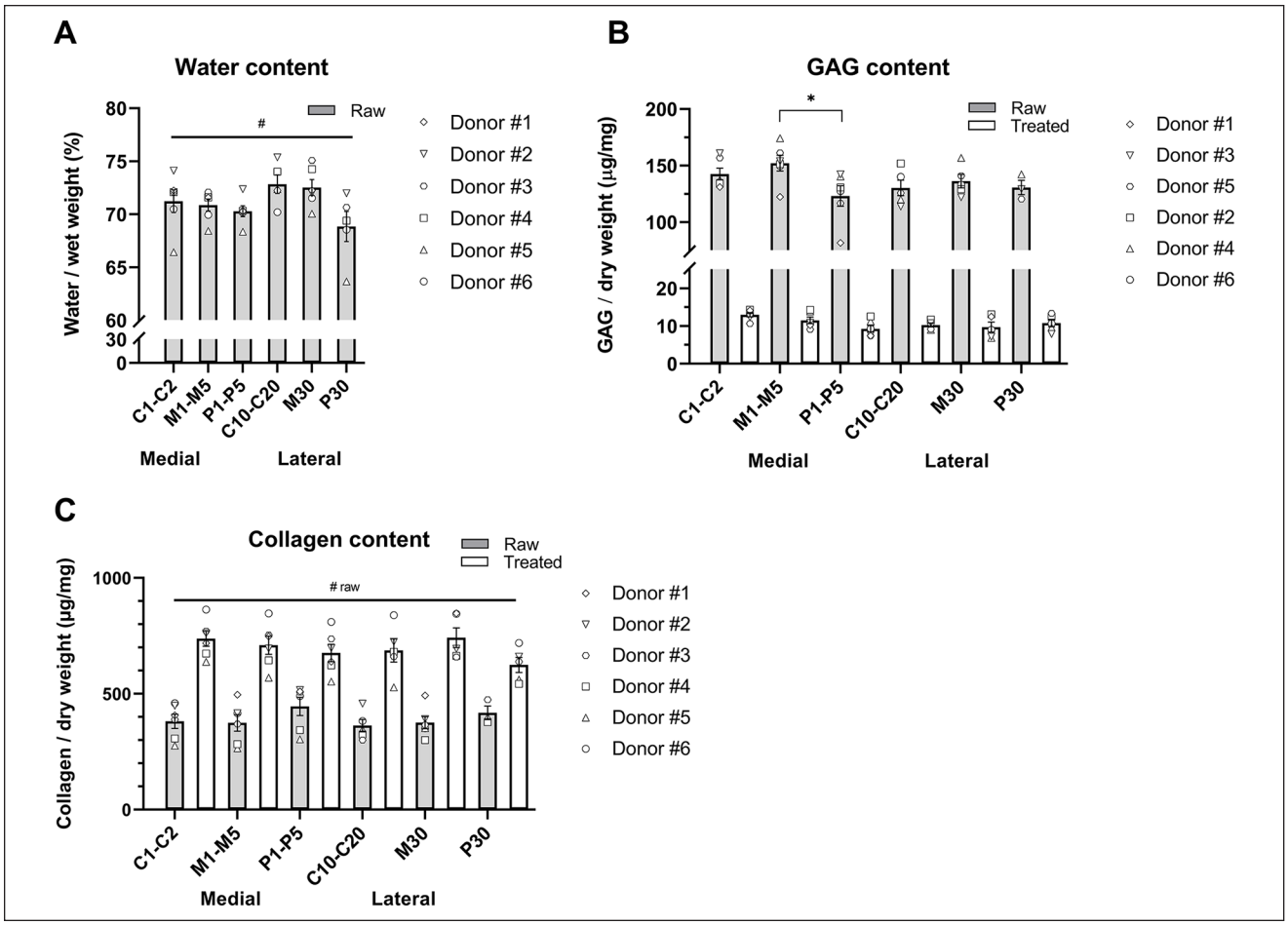

Figure 2. Tissue composition. The composition in raw (gray bars) and treated cartilage (open bars) for every areas on the plateau: Central (C1-C2), middle (M1-M5), and peripheral (P1-P5) on the medial condyle, and central (C10-C20), middle (M30), and peripheral (P30) on the lateral condyle. (**A**) Water content. (**B**) Glycosaminoglycan (GAG) content. (**C**) Collagen content. Values are mean of the areas for each donor, and error bars represent overall area mean ± SEM for all donors. #Equals $P < 0.05$ for analysis. *Equals $P < 0.05$ between areas.

during growth of collagen in this site corresponds to the atmospheric ^{14}C levels at that age. Thus, the actual collagen formation may have occurred during some years before and after the age of 7.5 years.

Estimated Age at Formation

A detailed overview with photos and estimated donor age at tissue formation for all donors is provided (Supplementary Fig. S2). The mean age was 11.7 ± 3.8 years (**Fig. 5A**). A schematic overview of the average age at formation for each anatomical location is presented for all donors together (**Fig. 5C**), showing collagen with ^{14}C levels corresponding to atmospheric levels present when the donors were 7 to 16 years of age. Between each condyle, the four samples at similar locations were compared (i.e., C1, C2, M3, and P3 medially vs. C10, C20, M30, and P30 laterally) showing higher age at formation medially at 11.43 ± 1.2 years than laterally at 10.33 ± 1.1 years ($P = 0.009$) (**Fig. 5B**).

Discussion

The Tibia Plateau Contains 2 Central Areas with Older Matrix and Expands Outward

We measured the lifelong incorporation of ^{14}C in enzymatically treated cartilage samples across the entire tibial condyle to characterize the developing growth pattern and timing of cartilage collagen formation. The enzymatically treated cartilage samples had high collagen content (70% ± 12%) and negligible GAG content (1% ± 0.3%) regardless of tibia location, and the ^{14}C data presented on the trypsin-resistant matrix are thus largely representative of fibrillar collagen. Our data demonstrate that the collagen at the center is generally oldest (5 out of 6 plateaus) and contains younger collagen radially outward toward the periphery.

Previous work showed a correlation between years at formation and distance to the edge of the tibia plateau regardless of OA,[10] and a cell-lineages tracking study by Kozhemyakina and colleagues[22] found that the greatest

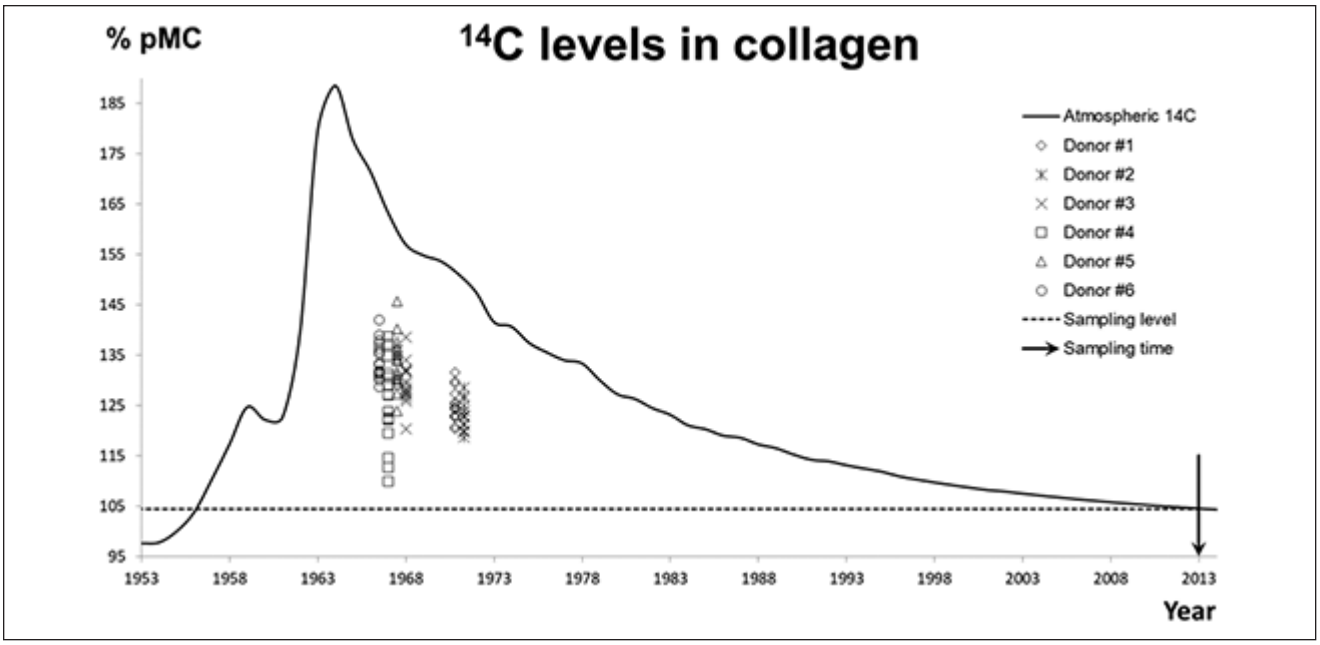

Figure 3. ^{14}C content in cartilage collagen. The ^{14}C bomb-pulse curve shows the chronological atmospheric concentration of ^{14}C (black line) as percent modern carbon (pMC). The horizontal dashed line indicates the approximate atmospheric ^{14}C level at the period of tissue sampling (February to December 2013). Vertically aligned symbols represent ^{14}C concentrations in treated cartilage from the same individual at the different locations on the tibia plateau. Several donors were born the same year, but for clarity the donors' birth years have been plotted months apart to separate the vertically aligned symbols.

apparent expansion of progenitors in the knee joint of mice occurred in the tibial articular cartilage, near the central/middle region of the condyles—that is, in the regions where this tissue experiences the highest levels of mechanical loading.[22] It could seem that mechanical loading by movement of the joint is a stimulus for the AC progenitor cells, and several groups speculate that a population of cells in the superficial zone have properties of stem cells that proliferate and expand laterally.[5,6,22-25] By applying cell-lineages tracking studies in mice, it has been observed that some superficial cells divided along the joint surface and then remained there, and further that superficial cells gave rise to clonal clusters, which might facilitate lateral cartilage expansion during juvenile growth.[23] Our data on the cartilage collagen matrix are in line with the data on cell proliferation with two central areas providing lateral expansion across each plateau. Unfortunately, due to methodological limitations it was not possible to separate superficial from more deep zones of the cartilage to provide zone-specific ^{14}C content measurements in the present study.

In addition to the central area being made first, we found strong trends suggesting that 2 concentric areas are developed radially outward. However, as one donor showed different growth pattern out of the limited number of donors in the current setup, further statistical significance was unattainable. Nonetheless, this intriguing finding would seem to indicate a childhood programming of cartilage formation and turnover. On the other hand, the opposite growth pattern found in one donor does demonstrate that an exception to the suggested programming is possible. This donor did experience major knee trauma at age 30 years (anamnestic information in patient history) leading to both a patella fracture and a lesion of the posterior cruciate ligament, which would lead to significant joint bleeding. This could induce cartilage changes as evidenced by hemophilic arthritis with frequent joint bleedings.[26] Furthermore, the posterior cruciate ligament lesion would change the mechanics in the joint and likely lead to different loading pattern on the medial condyle. As the growth on the lateral condyle (i.e., with less weight bearing) is similar to the pattern observed in the other donors, this further suggests a possible influence of changed mechanics due to the trauma. Since the pMC levels are very similar across the plateau (**Fig. 4A**), even minor turnover could tip the preprogrammed growth pattern to the one observed in this donor.

The Growth Pattern Forms within a Decade during School Years

The present study showed that the medial condyle contained slightly lower levels of ^{14}C corresponding to formation 1 year later than the lateral condyle. As the medial condyle is bigger, it could simply take longer to develop, alternatively, as it is loaded more, and as discussed above regarding cell behavior, mechanical loading could be a stimulus for formation, and the difference found between

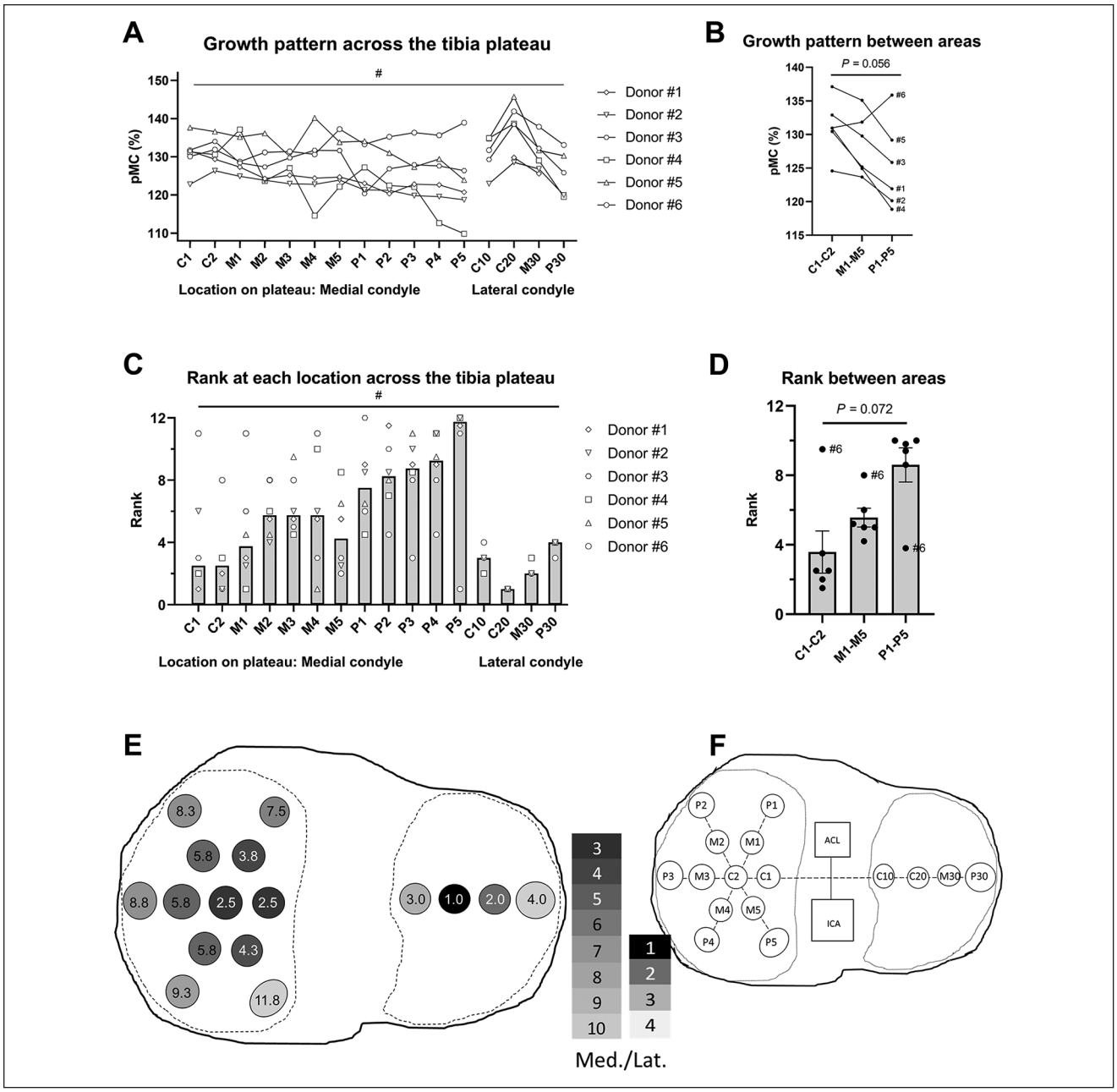

Figure 4. Growth pattern. (**A**) Tibia plateau growth pattern. Values are individual percent modern carbon (pMC) levels. (**B**) Growth pattern between central (C1-C2), middle (M1-M5), and peripheral (P1-P5) areas on the medial condyle. Values are mean pMC levels for each donor. (**C**) From each donor, the samples were ranked 1 to 12 on the medial condyle, and 1 to 4 on the lateral condyle using the mean rank for similar levels, with 1 being made first and 12 last. Values are individual ranks, and bars represent median rank for each location. (**D**) The mean ranks between central (C1-C2), middle (M1-M5), and peripheral (P1-P5) areas on the medial condyle. Values are mean ranks for each donor, and error bars represent mean rank for each area ± SEM. (**E**) Schematic presentation of the growth pattern on the tibia plateau. Values are median ranks at each location. (**F**) Location legend. ACL, anterior cruciate ligament; ICA, intercondylar area. #Equals $P < 0.05$ for analysis.

condyles could thus be due to mechanics. In tendons (also rich in fibrillar collagen), stable isotope infusion technique has indicated that a smaller fraction of collagen can be turned over more acutely in response to mechanical loading in adults.[27,28] To our knowledge, a loading effect specifically on collagen content or formation in cartilage has never been examined in children or in adolescents, and the implications for cartilage collagen made predominately at an age interval of 7 to 16 years are unclear. In young adult athletes aged 18 to 20 years, increased markers of collagen degradation were

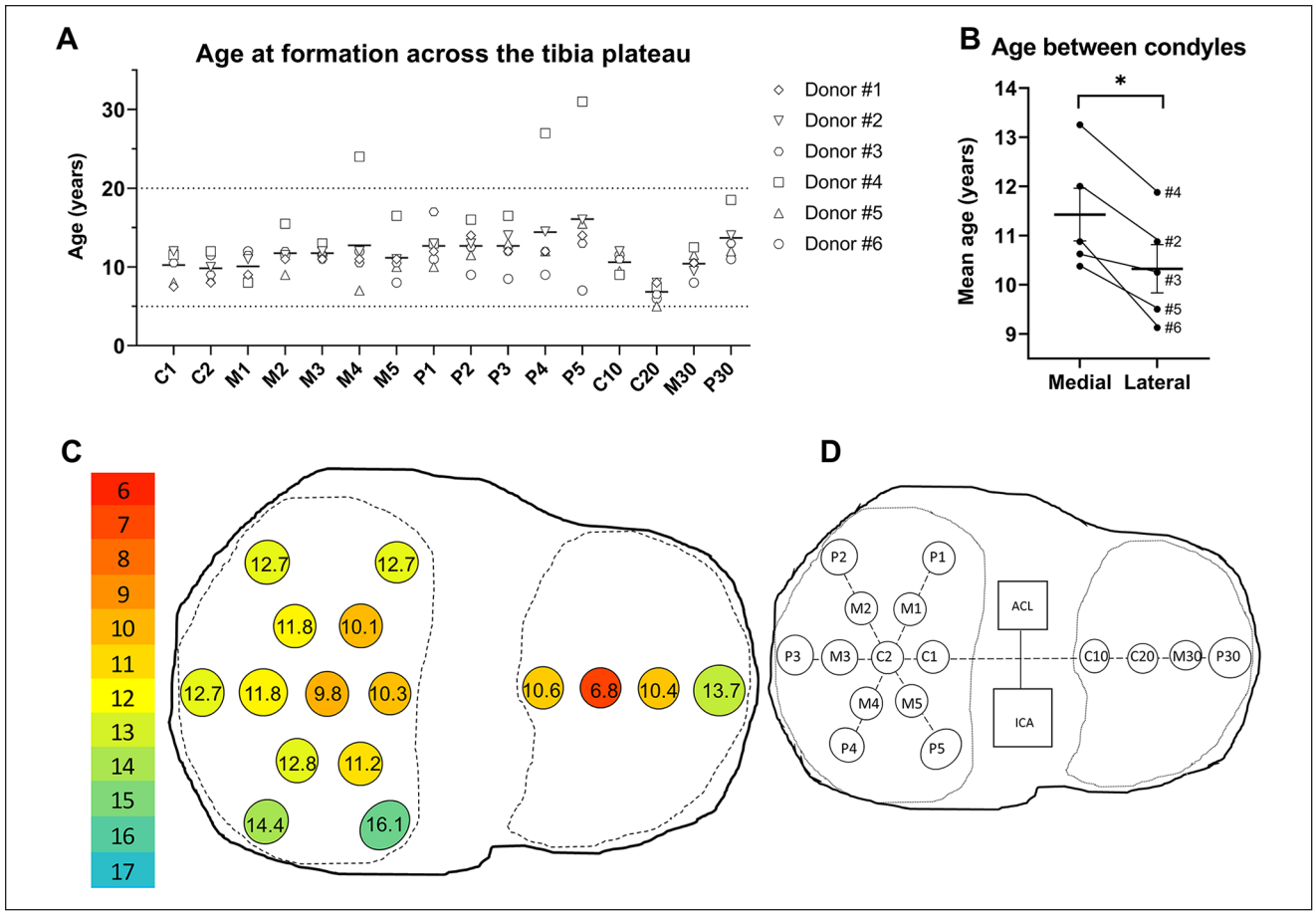

Figure 5. Age at formation. (**A**) Corresponding age at formation at every location. Values are individual data points in years, with lines representing the area means. (**B**) The age at formation between each condyle. Values are means in years of four samples on each condyle for each donor ($n = 5$ due to missing values in donor 1) and error bars represent condyle means ± SEM. (**C**) Schematic presentation of the age at formation on the tibia plateau. Values are mean years. (**D**) Location legend. ACL, anterior cruciate ligament; ICA, intercondylar area. *Equals $P < 0.05$.

found.[29] In school children, vigorous physical activity was associated to a greater amount of cartilage by magnetic resonance imaging scans, but did not provide details on cartilage composition.[30] However, when the same population of children was followed longitudinally, the association to physical activity became insignificant, possibly due to the small sample size of predominantly very active children.[31] Still, any effect on collagen by exercise or loading in childhood cannot be ruled out, although, in young animals and adult humans, the effect of exercise is primarily found on the GAGs and not on collagen itself.[32,33] We were not able to collect information regarding activity, however, when analyzing cartilage samples from donors over a large age-span, the ^{14}C levels do approximately follow the bomb-pulse curve.[10] This suggests that the donors follow similar turnover kinetics with only a small amount of collagen being turned over despite having different exercise/loading history. Considering these findings, it thus seems unlikely that any difference in activity levels between the donors would change the interpretation of the current data presented on collagen.

Cartilage Maintenance Potential in Adulthood

On average, we found that the trypsin-resistant matrix of cartilage collagen contained ^{14}C levels corresponding to levels in the atmosphere at donor age of 7 to 16 years with a mean age of 11.7 ± 3.8 years. This suggests very limited turnover in adulthood, where most of the collagen mass is retained after skeletal maturation. This confirms previous findings in cartilage by using ^{14}C levels[10] and by Banks *et al.*,[7] who showed accumulation of advanced glycation end-products (AGEs) after the age of 20 years in human femoral (hip) cartilage. As AGEs are nonenzymatic cross-links only removed by tissue breakdown, AGEs accumulate in tissues with low turnover. However, as shown in **Figure 5A**, some samples in one donor contained pMC levels corresponding to a formation age around 30 years. Although

the peripheral areas in this donor were macroscopically colored violet, they were not osteophytes, which is a fibrocartilaginous outgrowth containing newer matrix.[34] Because the plateau contained darkened areas of cartilage in the periphery, an extra sample was taken (Supplementary Fig. S2: white circle on photo) to confirm the high values in this posterior part of the medial plateau. Consistently with the surrounding biopsies, this had an age at formation at 39 years. Thus, it appears that factors can intervene resulting in regional matrix incorporation of newer carbon later in life. By chance, we were able to capture this unique phenomenon, although it must be stressed that none of the other donors in the current nor the previous study[10] showed any sign of newer collagen synthesis. Finally, due to discoloration of the plateau, we cannot rule out an unidentified underlying condition.

As the measured pMC level is a mass-weighted average of each sample biopsy, minor amounts of newer collagen could be incorporated into the matrix throughout life, and thus, it cannot be concluded that any collagen syntheses later in life is impossible. Furthermore, cartilage could be maintained by other parts of the matrix not analyzed in the current setup. In mice, it has been demonstrated that the persistent collagen mesh of incorporated collagen fibers experience rhythmic circadian maintenance from a pool of newly synthesized soluble collagen,[35] and a recent study in human cartilage not using enzymatic treatment did show soluble collagen type II turnover.[36] Our results are on the trypsin-resistant collagen matrix, and any new unincorporated collagen would not have been measured, but removed by the extraction protocol. Thus, a steady synthesis of soluble collagen not incorporated in the cartilage matrix cannot be ruled out. Furthermore, collagen maturations with cross-links could still influence the cartilage strength later in life. However, the ^{14}C method cannot provide answers regarding cross-links, as carbon from the collagen helixes greatly outnumber carbon found in cross-links. Finally, a recent study using continuous infusion of stabile isotopes hours prior to knee replacement surgery showed turnover in raw cartilage,[37] and this result is therefore likely to be carried by proteins with a short half-life, for example, proteoglycans. Thus, future studies could use isotope infusion over a longer period combined with enzymatic treatment of cartilage to examine if the stable matrix of predominately collagen does indeed have any turnover.

Cartilage Turnover in Early Childhood (Preschool)

In the present study, we did not find any samples containing ^{14}C levels corresponding to atmospheric levels present before a donor age of 5 years, which indicates that the cartilage matrix studied is either completely renewed, or that the samples contain embryonic collagen mixed with collagen formed later in life. Both would still lead to the average ^{14}C levels found in the samples. In tendons, it has been shown that during embryonic development, the collagen fibrils increase in both number and length, but during postnatal growth they remain at a constant fibril number, of increasing length and diameter.[38] This could imply that the collagen fibrils are made during embryogenesis and not replaced, but instead, newer collagen is made during growth. Postnatal collagen could also be formed by remodeling, meaning that the embryonic fibrils are replaced by thicker and longer collagen fibrils. Our data fit both possibilities, as an addition of new collagen or gradual replacement of embryonic collagen would lead to a dilution of the embryonic fibrils' ^{14}C levels resulting in ^{14}C levels corresponding to atmospheric levels some years after birth as found in the current study—though some or even all the embryonic collagen might remain.

In addition to the overall common finding in the different cartilage donors, clearly individual differences were present.

In this current explorative study, we are limited by a relatively low number of donors with different clinical conditions, and additional donors would have provided more strength to the analyses.

In conclusion, by using the bomb pulse our data show that human cartilage on the tibia plateau contains collagen that has experienced little turnover since adolescence. Furthermore, the tibia plateau develops centrally on each condyle, expanding radially outward, with the lateral condyle made slightly earlier than the medial. We suggest a possibility for a childhood programmed cartilage formation, and a very limited adult turnover kinetic only partly susceptible to minor increases with disease.

Author Contributions

AEMJ, PS, and KMH participated in all phases of the experiment. MK participated in the planning of the study and data interpretation. AEMJ performed the laboratory work and drafted the initial manuscript. MRK and MMP collected the tissue. JO was responsible for radiocarbon and stable isotope analysis. All authors reviewed, edited, and approved the final version of the manuscript. AEMJ and KMH take responsibility for the integrity of the work as a whole.

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Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Ethical Approval

The study was conducted in accordance to the Declaration of Helsinki, and ethical approval for obtaining waste tissue was obtained from the Ethical Committee of the Capital Region of Denmark (H-4-2012-131).

Informed Consent

All participants gave written informed consent.

Trial Registration

Not applicable.

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References

1. Jay GD, Waller K a. The biology of lubricin: near frictionless joint motion. Matrix Biol. 2014;39:17-24. doi:10.1016/j.matbio.2014.08.008
2. Kronenberg HM. Developmental regulation of the growth plate. Nature. 2003;423(6937):332-6. doi:10.1038/nature01657
3. Decker RS, Um HB, Dyment NA, Cottingham N, Usami Y, Enomoto-Iwamoto M, Kronenberg MS, *et al*. Cell origin, volume and arrangement are drivers of articular cartilage formation, morphogenesis and response to injury in mouse limbs. Dev Biol. 2017;426(1):56-68. doi:10.1016/j.ydbio.2017.04.006
4. Dudhia J. Aggrecan, aging and assembly in articular cartilage. Cell Mol Life Sci. 2005;62(19-20):2241-56. doi:10.1007/s00018-005-5217-x
5. Hunziker EB, Kapfinger E, Geiss J. The structural architecture of adult mammalian articular cartilage evolves by a synchronized process of tissue resorption and neoformation during postnatal development. Osteoarthritis Cartilage. 2007;15(4):403-13. doi:10.1016/j.joca.2006.09.010
6. Hayes AJ, MacPherson S, Morrison H, Dowthwaite G, Archer CW. The development of articular cartilage: evidence for an appositional growth mechanism. Anat Embryol (Berl). 2001;203(6):469-79. doi:10.1007/s004290100178
7. Bank RA, Bayliss MT, Lafeber FP, Maroudas A, Tekoppele JM. Ageing and zonal variation in post-translational modification of collagen in normal human articular cartilage. The age-related increase in non-enzymatic glycation affects biomechanical properties of cartilage. Biochem J. 1998;330 (Pt 1):345-51.
8. Julkunen P, Harjula T, Iivarinen J, Marjanen J, Seppänen K, Närhi T, *et al*. Biomechanical, biochemical and structural correlations in immature and mature rabbit articular cartilage. Osteoarthritis Cartilage. 2009;17(12):1628-38. doi:10.1016/j.joca.2009.07.002
9. Rieppo J, Hyttinen MM, Halmesmaki E, Ruotsalainen H, Vasara A, Kiviranta I, *et al*. Changes in spatial collagen content and collagen network architecture in porcine articular cartilage during growth and maturation. Osteoarthritis Cartilage. 2009;17(4):448-55. doi:10.1016/j.joca.2008.09.004
10. Heinemeier KM, Schjerling P, Heinemeier J, Møller MB, Krogsgaard MR, Grum-Schwensen T, *et al*. Radiocarbon dating reveals minimal collagen turnover in both healthy and osteoarthritic human cartilage. Sci Transl Med. 2016;8(346):346ra90. doi:10.1126/scitranslmed.aad8335
11. Verzijl N, DeGroot J, Thorpe SR, Bank RA, Shaw JN, Lyons TJ, *et al*. Effect of collagen turnover on the accumulation of advanced glycation end products. J Biol Chem. 2000;275(50):39027-31. doi:10.1074/jbc.M006700200
12. Maroudas A, Palla G, Gilav E. Racemization of aspartic acid in human articular cartilage. Connect Tissue Res. 1992;28(3):161-9.
13. Goodsite ME, Rom W, Heinemeier J, Lange T, Ooi S, Appleby PG, *et al*. High-resolution AMS 14C dating of post-bomb peat archives of atmospheric pollutants. Radiocarbon. 2001;43(2B):495-515.
14. Lynnerup N, Kjeldsen H, Heegaard S, Jacobsen C, Heinemeier J. Radiocarbon dating of the human eye lens crystallines reveal proteins without carbon turnover throughout life. PLoS One. 2008;3(1):e1529. doi:10.1371/journal.pone.0001529
15. Schmidt MB, Mow VC, Chun LE, Eyre DR. Effects of proteoglycan extraction on the tensile behavior of articular cartilage. J Orthop Res. 1990;8(3):353-63. doi:10.1002/jor.1100080307
16. Rosenblum G, Van den Steen PE, Cohen SR, Bitler A, Brand DD, Opdenakker G, *et al*. Direct visualization of protease action on collagen triple helical structure. PLoS One. 2010;5(6):e11043. doi:10.1371/journal.pone.0011043
17. Olsen J, Tikhomirov D, Grosen C, Heinemeier J, Klein M. Radiocarbon analysis on the new AARAMS 1MV tandetron. Radiocarbon. 2017;59(3):905-13. doi:10.1017/RDC.2016.85
18. Stuiver M, Polach HA. Discussion reporting of 14C data. Radiocarbon. 1977;19(3):355-63. doi:10.1017/S0033822200003672
19. Neuman RE, Logan MA. The determination of hydroxyproline. J Biol Chem. 1950;184(1):299-306.
20. Kueppers LM, Southon J, Baer P, Harte J. Dead wood biomass and turnover time, measured by radiocarbon, along a subalpine elevation gradient. Oecologia. 2004;141(4):641-51. doi:10.1007/s00442-004-1689-x
21. Levin I, Kromer B, Hammer S. Atmospheric $\Delta^{14}CO_2$ trend in Western European background air from 2000 to 2012. Tellus B Chem Phys Meteorol. 2013;65(1):20092. doi:10.3402/tellusb.v65i0.20092
22. Kozhemyakina E, Zhang M, Ionescu A, Ayturk UM, Ono N, Kobayashi A, *et al*. Identification of a Prg4 -expressing articular cartilage progenitor cell population in mice. Arthritis Rheumatol. 2015;67(5):1261-73. doi:10.1002/art.39030
23. Li L, Newton PT, Bouderlique T, Sejnohova M, Zikmund T, Kozhemyakina E, *et al*. Superficial cells are self-renewing chondrocyte progenitors, which form the articular cartilage in

juvenile mice. FASEB J. 2017;31(3):1067-84. doi:10.1096/fj.201600918R
24. Dowthwaite GP, Bishop JC, Redman SN, Khan IM, Rooney P, Evans DJR, *et al*. The surface of articular cartilage contains a progenitor cell population. J Cell Sci. 2004;117(Pt 6):889-97. doi:10.1242/jcs.00912
25. Hattori S, Oxford C, Reddi AH. Identification of superficial zone articular chondrocyte stem/progenitor cells. Biochem Biophys Res Commun. 2007;358(1):99-103. doi:10.1016/j.bbrc.2007.04.142
26. Roosendaal G, Lafeber FP. Pathogenesis of haemophilic arthropathy. Haemophilia. 2006;12(Suppl 3):117-21. doi:10.1111/j.1365-2516.2006.01268.x
27. Heinemeier KM, Schjerling P, Heinemeier J, Magnusson SP, Kjaer M. Lack of tissue renewal in human adult Achilles tendon is revealed by nuclear bomb (14)C. FASEB J. 2013;27(5):2074-9. doi:10.1096/fj.12-225599
28. Miller BF, Olesen JL, Hansen M, Døssing S, Crameri RM, Welling RJ, *et al*. Coordinated collagen and muscle protein synthesis in human patella tendon and quadriceps muscle after exercise. J Physiol. 2005;567(Pt 3):1021-33. doi:10.1113/jphysiol.2005.093690
29. O'Kane JW, Hutchinson E, Atley LM, Eyre DR. Sport-related differences in biomarkers of bone resorption and cartilage degradation in endurance athletes. Osteoarthritis Cartilage. 2006;14(1):71-6. doi:10.1016/j.joca.2005.08.003
30. Jones G, Glisson M, Hynes K, Cicuttini F. Sex and site differences in cartilage development: a possible explanation for variations in knee osteoarthritis in later life. Arthritis Rheum. 2000;43(11):2543-9. doi:10.1002/1529-0131(200011)43:11<2543::AID-ANR23>3.0.CO;2-K
31. Jones G, Ding C, Glisson M, Hynes K, Ma D, Cicuttini F. Knee articular cartilage development in children: a longitudinal study of the effect of sex, growth, body composition, and physical activity. Pediatr Res. 2003;54(2):230-6. doi:10.1203/01.PDR.0000072781.93856.E6
32. Jørgensen AEM, Kjær M, Heinemeier KM. The effect of aging and mechanical loading on the metabolism of articular cartilage. J Rheumatol. 2017;44(4):410-7. doi:10.3899/jrheum.160226
33. Bricca A, Juhl CB, Grodzinsky AJ, Roos EM. Impact of a daily exercise dose on knee joint cartilage—a systematic review and meta-analysis of randomized controlled trials in healthy animals. Osteoarthritis Cartilage. 2017;25(8):1223-37. doi:10.1016/j.joca.2017.03.009
34. van der Kraan PM, van den Berg WB. Osteophytes: relevance and biology. Osteoarthritis Cartilage. 2007;15(3):237-44. doi:10.1016/j.joca.2006.11.006
35. Chang J, Garva R, Pickard A, Yeung CYC, Mallikarjun V, Swift J, *et al*. Circadian control of the secretory pathway maintains collagen homeostasis. Nat Cell Biol. 2020;22(1):74-86. doi:10.1038/s41556-019-0441-z
36. Hsueh MF, Önnerfjord P, Bolognesi MP, Easley ME, Kraus VB. Analysis of "old" proteins unmasks dynamic gradient of cartilage turnover in human limbs. Sci Adv. 2019;5(10):eaax3203. doi:10.1126/sciadv.aax3203
37. Smeets JSJ, Horstman AMH, Vles GF, Emans PJ, Goessens JPB, Gijsen AP, *et al*. Protein synthesis rates of muscle, tendon, ligament, cartilage, and bone tissue in vivo in humans. PLoS One. 2019;14(11):e0224745. doi:10.1371/journal.pone.0224745
38. Kalson NS, Lu Y, Taylor SH, Starborg T, Holmes DF, Kadler KE. A structure-based extracellular matrix expansion mechanism of fibrous tissue growth. Elife. 2015;4:e05958. doi:10.7554/eLife.05958

GRADUATE SCHOOL OF HEALTH AND MEDICAL SCIENCES
UNIVERSITY OF COPENHAGEN

PHD-THESIS
DECLARATION OF CO-AUTHORSHIP

The declaration is for PhD students and must be completed for each conjointly authored article. Please note that if a manuscript or published paper has ten or less co-authors, all co-authors must sign the declaration of co-authorship. If it has more than ten co-authors, declarations of co-authorship from the corresponding author(s), the senior author and the principal supervisor (if relevant) are a minimum requirement.

1. Declaration by	
Name of PhD student	Adam El Mongy Jørgensen
E-mail	adam.em.jorgensen@gmail.com
Name of principal supervisor	Michael Kjær
Title of the PhD thesis	Human cartilage growth, regional turnover in vivo, and the effect of exercise in late-stage knee osteoarthritis

2. The declaration applies to the following article	
Title of article	Collagen Growth Pattern in Human Articular Cartilage of the Knee.
Article status	
Published ☐ Date:	Accepted for publication ☐ Date:
Manuscript submitted ☒ Date: 4th of June 2020	Manuscript not submitted ☐
If the article is published or accepted for publication, please state the name of journal, year, volume, page and DOI (if you have the information).	

3. The PhD student's contribution to the article ***(please use the scale A-F as benchmark)*** Benchmark scale of the PhD-student's contribution to the article **A.** Has essentially done all the work (> 90 %) **B.** Has done most of the work (60-90 %) **C.** Has contributed considerably (30-60 %) **D.** Has contributed (10-30 %) **E.** No or little contribution (<10 %) **F.** Not relevant	**A, B, C, D, E, F**
1. Formulation/identification of the scientific problem	D
2. Development of the key methods	F
3. Planning of the experiments and methodology design and development	C
4. Conducting the experimental work/clinical studies/data collection/obtaining access to data	B
5. Conducting the analysis of data	B
6. Interpretation of the results	B
7. Writing of the first draft of the manuscript	A
8. Finalisation of the manuscript and submission	A
Provide a short description of the PhD student's specific contribution to the article.[i] On already collected cartilage speciments, the PhD student planned and performed all the lab work: Systematic location and sampling of biopsies, enzymatic treatments, and assays for matrix components. Further, the PhD student performed statistics with co-authors, made all the figures, wrote the initial manuscript, and incorporated comments and feedback from co-authors into the final manuscript.	

4. Material from another thesis / dissertation[ii]	
Does the article contain work which has also formed part of another thesis, e.g. master's thesis, PhD thesis or doctoral dissertation (the PhD student's or another person's)?	Yes: ☐ No: ☒
If yes, please state name of the author and title of thesis / dissertation.	N/A
If the article is part of another author's academic degree, please describe the PhD student's and the author's contributions to the article so that the individual contributions are clearly distinguishable from one another.	N/A

5. Signatures of the co-authors[iii]

	Date	Name	Title	Signature
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10.				

6. Signature of the principal supervisor

I solemnly declare that the information provided in this declaration is accurate to the best of my knowledge.
Date: 11/8 20
Principal supervisor: Michael Kjær

7. Signature of the PhD student

I solemnly declare that the information provided in this declaration is accurate to the best of my knowledge.
Date:
PhD student: Adam El Mongy Jørgensen 11/8-20

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[i] This can be supplemented with an additional letter if needed.
[ii] Please see Ministerial Order on the PhD Programme at the Universities and Certain Higher Artistic Educational Institutions (PhD Order) § 12 (4):
"Any articles included in the thesis may be written in cooperation with others, provided that each of the co-authors submits a written declaration stating the PhD student's or the author's contribution to the work."
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The regional turnover of cartilage collagen matrix in late-stage human knee osteoarthritis

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ABSTRACT **Background:** Osteoarthritis (OA) affects millions worldwide with an increasing incidence. Cartilage collagen has very limited repair potential, however, some turnover and incorporation has not been fully excluded. The aim of this study was to determine the regional turnover of human OA cartilage.

Methods: Patients scheduled for knee joint replacement surgery due to OA were recruited in this prospective study of four weeks. Deuterium oxide (heavy water, D_2O) was administered orally by weekly boluses initially 150 ml (70%) followed by three boluses of 50 ml (70%). Cartilage from the medial tibia plateau/condyle was sampled 1) centrally, 2) under the meniscus, and 3) from osteophytes, and all treated enzymatically with hyaluronidase and trypsin. Samples were analysed for deuterium incorporation using mass spectrometry and for gene expression by real-time reverse transcriptase polymerase chain reaction.

Results: Twenty patients completed the study: mean age 64±9.1 years (SD), 45% female, BMI 29.5±4.8 kg/m² (SD) providing a total of 21 plateaus (one had bilateral surgery). Treated cartilage from both condylar regions showed similar enrichments of 0.063% APE, while osteophytes showed significantly increased enrichment of 0.072% APE ($P < 0.001$). Regionally, submeniscal areas had increased gene expression of MMP-3 and decreased lubricin expression compared to central. Untreated samples had increased enrichments of 0.075% APE compared to treated at 0.065% APE.

Conclusion: In OA, despite regional differences in gene expression, the turnover of the articular cartilage matrix across the entire joint surface is limited, and only slightly higher in osteophyte cartilage.

Keywords: Deuterium, gene expression, mass spectrometry.

INTRODUCTION

Articular cartilage (AC) contains a single cell type, the chondrocyte, surrounded by an extracellular matrix composed of a fibrillary network of both collagens and non-collagenous matrix components embedded in a gel-like substance. The collagen network content comprises 60% of dry weight [1] with the large majority being type II collagen (>90 %) and small amounts of type VI, IX and XI collagen[2,3]. Osteoarthritis (OA) is a painful and debilitating disease of the joint[4], which occurs with an increasing frequency with ageing[5]. One of the hallmarks of OA is cartilage degradation, due to increased catabolism where the proteoglycans and glycosaminoglycans (GAGs) are removed, thereby exposing the underlying collagen fibres for possible irreversible breakdown mainly by matrix metalloproteinase 13 (MMP-13)[6]. As cartilage sampling is highly invasive and destructive, biomarkers for assessing *in vivo* collagen turnover have been used with unclear results[7]. Further, the biomarkers target the cleaved-off N-terminal and C-terminal propeptides, and thus, do not reflect collagen incorporation but only synthesis. *Ex-vivo* models allow treatment of cartilage to perform measurements on the collagen fibres, which has showed a half-life >100 years using rate of racemization of aspartic acid or accumulation of advanced glycation end-products[8,9]. However, as these methods are influenced by physical and chemical factors i.e. pH and temperature[10,11], tracing incorporation of isotopes has been used. In human tibia cartilage, life-long incorporation of ^{14}C shows collagen to be without any major incorporation after the school years despite the presence of OA[12]. This

supports the notion that collagen fibre degradation is irreversible, yet increased incorporation of radioactive tracers in OA compared to healthy has been found *in vitro* from enzymatic treated human cartilage[13,14] and *in vivo* from canine cartilage[15]. Conditions vary greatly between *in vivo* and *in vitro*[16], and the very rapid progression of surgically induced OA in an animal model is quite different from the naturally occurring human OA[7]. Nevertheless, when measuring ^{14}C, a mass-weighted average is reported, and therefore, minor amounts of newer collagen could in fact be incorporated into the collagen matrix. Thus, it is unknown whether some potential for renewability of the incorporated collagen matrix does indeed exists in humans with OA, which would be vital for cartilage conservation and integrity. Therefore, we aim to examine the turnover of incorporated collagen *in vivo* from humans with OA. We hypothesise that the isotope incorporation into cartilage will be minor.

METHODS

Study design

Four weeks prior to scheduled surgery, 20 patients orally ingested deuterium oxide, D_2O (^2H_2O 99.8% Cambridge Isotope Laboratories Inc., Andover, MA, USA) once a week, and blood samples were taken to measure the enrichment of labelled amino acids (Fig. 1). D_2O was administered weekly with a bolus 150 ml (70%) four weeks prior and boluses the following three weeks of 50 ml (70%) using modified and recommended approaches by others albeit not measuring on cartilage tissue[17,18]. The blood sample at two hours after initial ingestion was chosen as the expected time-point of maximum body water enrichment[19]. The study was performed on Copenhagen University Hospital of Bispebjerg in Denmark. Participants gave written informed content and the study was approved by the Ethical Committee of the Capital Region of Denmark (H-17015563) complying with the Helsinki Declaration II. The trial was registered on ClinicalTrials.gov (NCT03410745).

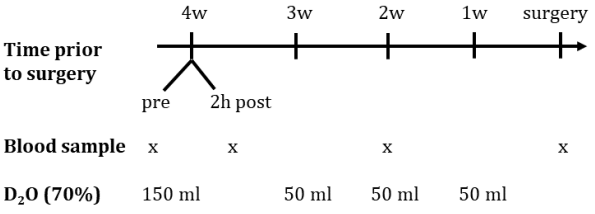

Figure 1: Schematic representation of study protocol
The study protocol is initiated four weeks (4w) prior to the scheduled knee replacement surgery. The blood samples are drawn before administering deuteriated water at 70% atom concentration.

Participants

Patients were recruited at the University Hospital from the outpatient clinic of the orthopaedic department. Patients with OA of the knee as classified by the American College of Rheumatology[20] scheduled for a knee replacement surgery by an orthopaedic surgeon were considered for participation. Patients were eligible if they were 50–90 years old, had primary OA, had a body mass index (BMI) within 18.5–40 kg/m², were non-smoker, tracer naïve, and willing to schedule their surgery later than four weeks (the treatment guarantee is one month in Denmark) to allow for the labelling time.

Exclusion criteria included other diseases of the joint (rheumatoid arthritis, gout, or psoriasis arthritis), systemic inflammatory diseases, diabetes or cancer, previous knee surgery or recent trauma to the knee, participation in regular strenuous exercise, oral or intraarticular injection of corticoid-steroids within the last three months, or requirement of an interpreter.

Tissue collection

During knee replacement surgery, the leftover tibia plateau was collected and immediately transferred to the laboratory on ice. From the medial tibia condyle, several full-thickness cartilage biopsies were sampled centrally and under the meniscus. By cutting with a scalpel, the subchondral bone was removed from biopsies. From osteophytes (if present), slices were sampled using a scalpel. All cartilage for messenger ribonucleic acid (mRNA) extraction was snap-frozen in liquid nitrogen and kept on −80°C until further analysis, while cartilage for isotope analysis was weighted, cut in thin sections (<1 mm) perpendicular to the surface, freeze-dried, and weighted again.

Blood samples were collected in K_3-EDTA plasma tubes (Vacuette, Greiner Bio-One, Kremsmünster, Austria). After resting for 30 minutes on ice, the tube was centrifuged for 10 minutes at 4°C at 3172 G before the plasma fraction was collected, gently mixed, aliquoted, and kept on −80°C until further analysis.

Collagen purification

To determine the incorporation in collagen, GAGs and other none-collagenous substances were removed using a collagen extraction procedure as previously described[12]. Six samples of osteophyte cartilage were of sufficient size

for keeping a part untreated. Freeze-dried samples were treated overnight with hyaluronidase (H3506, Sigma) [5 U/ml in 0.05 M sodium acetate and 0.15 M NaCl (pH=6)] at 37°C. The samples were centrifuged, the supernatant removed, and the cartilage slices were washed with isotonic NaCl before being incubated with trypsin (T8802, Sigma) [1 mg/ml in PBS] overnight at 37°C. The samples were centrifuged, the supernatant removed, and the samples were washed with PBS, 0.7 M KCl, and distilled water. Finally, samples were freeze-dried, weighed, and kept at −80°C for later analyses. As trypsin is not able to cleave native triple helical collagen[21], our protocol would remove any unincorporated collagen and leave behind an indigestible cartilage matrix of predominately fibrillar collagen type II.

Isotope analyses

The analyses were performed as described elsewhere[22]. In brief, precipitated plasma proteins and enzymatic treated cartilage samples were hydrolysed in 6M HCl at 110°C overnight to liberate the amino acids. Plasma samples for free amino acids were acidified. All samples were then purified on cation exchange resin columns (Bio-Rad, Hercules, CA, USA), liberated using 2M NH_4OH, and dried by N_2 flow at 70°C. Finally, the amino acids were derivatised to their corresponding N-acetyl-N-propyl (NAP) esters for analyses. The amount of deuterium labelled alanine (D-ala) was analysed using gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS) and free plasma enrichment was analysed by liquid chromatography isotope ratio mass spectrometry (LC-MS/MS) systems (Finnigan, Bremen, Germany). The $^2H/^1H$ ratio was converted to atom percent excess (APE) using the absolute ratio constant for deuterium based on the VSMOW standard, as described elsewhere[23].

Gene expression analysis

Cartilage biopsies were cut in 10 µm slices at −20°C. The slices were homogenised in TriReagent (Molecular Research Center, Cincinnati, OH, USA) using 2.3 mm stainless steel beads in a homogenising tube (BioSpec Products Inc., Bartlesville, OK, USA) and shaken (FastPrep, FP Biomedicals, Illkirch, France). Bromo-chloropropane (Sigma, Darmstadt, Germany) was added to isolate RNA containing aqueous phase, where a poly-acryl carrier and isopropanol were added to precipitate the mRNA. The pellet was washed with 75% ethanol, dissolved in water, transferred to spin columns (Quiagen, Hilden, Germany) for further washing, and then finally eluted with water. The RNA concentration was determined by the RiboGreen assay (Molecular Probes Inc., Eugene, OR, USA) as per manufacture's protocol, which uses a fluorescent nucleic acid stain for quantitating RNA from a standard curve. The extracted mRNA was then converted to 50 ng cDNA using poly-dT (Invitrogen, Nærum, Denmark) with the OmniScript Kit (Qiagen, Hilden, Germany) as per manufacture's protocol. Real-time RT-PCR (Stratagene, La Jolla, CA, USA) with specific primers (table S1) were performed using Ct values from a standard curve of DNA oligos (Ultramer, Integrated DNA Technologies Inc., Leuven, Belgium) with a DNA sequence corresponding to the expected PCR product. The specificity of the PCR products was confirmed by melt curves of unknown samples and known oligos. Expressed values were normalised to internal control of large ribosomal protein Po (RPLPo), which was then validated using glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Statistics

All statistical analyses were performed using GraphPad Prism version 8.4.1 (GraphPad Software, San Diego, CA, USA). For cartilage differences with location, Friedman test was used with Dunn's multiple comparisons test post hoc. RNA targets were analysed by paired t-test on log values. Statistical significance was defined as *P*-values < 0.05 for all analyses. Values are reported as medians with interquartile range, and geometric means ± back transformed SEM for gene expression data.

RESULTS

Patients were recruited for participation between March 2018 and October 2019 (Fig. 2). Twenty participants completed the intervention: mean age 64±9.1 years (SD), 45% female, BMI 29.5±4.8 kg/m² (SD) providing 21 tibia plateaus (table 1) as one participant had bilateral surgery. After ingestion of the first bolus only, three participants reported slight light-headedness but not true vertigo, which lasted between 20–45 minutes.

Blood enrichment

Plasma precursor enrichment of free D-ala showed the highest median enrichment of 0.65% APE with interquartile range of [0.55–0.78] two hours following the initial 150 ml bolus, and remained relatively stable, as two weeks prior to surgery (the time-point expected to contain the lowest precursor enrichment) showed a median enrichment of 0.40% APE [0.29-0.45]. Thus, comparable to others using a similar setup[17], a relatively

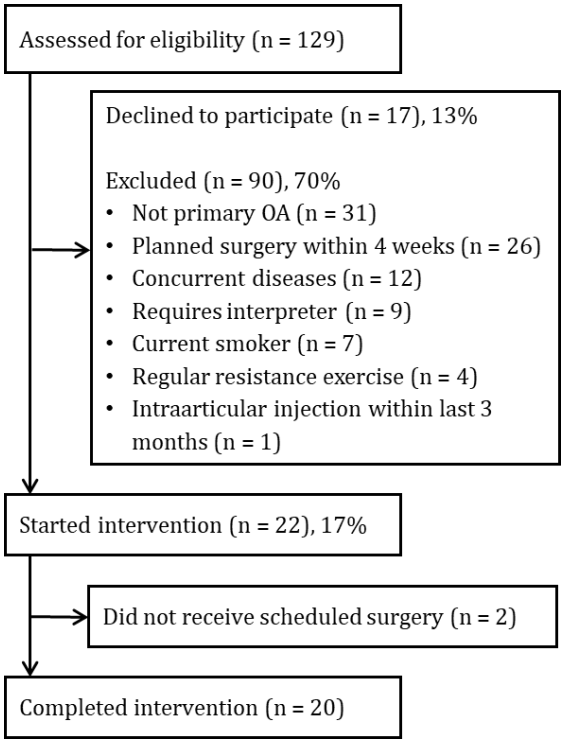

Figure 2: Participant flow chart
The flow of participants. One donor had bilateral surgery, and thus 21 tibia plateaus were collected.

Table 1: Participants' characteristics.

Parameter	Value
Age (years)	64.5 ± 9.1
Body mass index (kg/m²)	29.5 ± 4.8
Sex (female:male)	9:11
Kellgren-Lawrence grade (0-4)	3.5 ± 0.5
Labelling duration (days)	28.1 ± 1.1
Daily medication intake	
Analgesics	12 (60%)
Acetaminophen/paracetamol	10
NSAID (ibuprofen or naproxen)	8
Opioids (tramadol or morphine)	2
Anti-thrombotic	4 (20%)
Anti-hypertensive	8 (40%)
Anti-hyperlipidemic	4 (20%)

NSAID: non-steroid anti-inflammatory drug. Values are means ± SD or the number of participants and the percentage.

stable range during the labelling period can be assumed albeit with a minimum of sampling time-points. As a positive control of deuterium labelling, we used plasma protein D-ala enrichment at the time of surgery, which showed a median enrichment of 0.18% APE [0.17–0.20] (Fig. 3A). Thus, it could be validated that oral ingestion of heavy water led to deuterium-labelled alanine being incorporated into proteins.

Cartilage turnover

Treated cartilage showed similar median D-ala of 0.063 [0.062-0.063] % APE from both central and submeniscal regions, while osteophytes showed significantly higher enrichment of 0.072% [0.066-0.085] APE ($P < 0.001$) compared to either central or submeniscal region (Fig. 3B). Paired treated and untreated samples of central (n=2), submeniscal (n=2), and osteophyte (n=6) cartilage showed a median D-ala of 0.075 [0.071-0.078] % APE in untreated samples, which was consistently higher than the D-ala of 0.065 [0.062-0.070] % APE in treated samples (Fig. 4).

Gene-expression

We did not see any difference in GAPDH, thus validating the use of RPLP0 for normalisation (Fig. 5). Collagen type II (COL2A1), aggrecan (ACAN), lubricin (PRG4), and matrix metalloproteinase 3 (MMP-3) genes were detected sufficiently for analyses (Fig. 5), however, MMP-1, MMP-13, and a disintegrin and metalloproteinase with thrombospondin motifs 5 (ADAMTS-5) were detected in too low amounts, and ADAMTS-4 was undetected. When compared to central area, submeniscal areas had increased MMP-3 by 4.1–fold ($P < 0.0001$) and decreased lubricin expression by 0.6–fold ($P = 0.0443$) (Fig. 5).

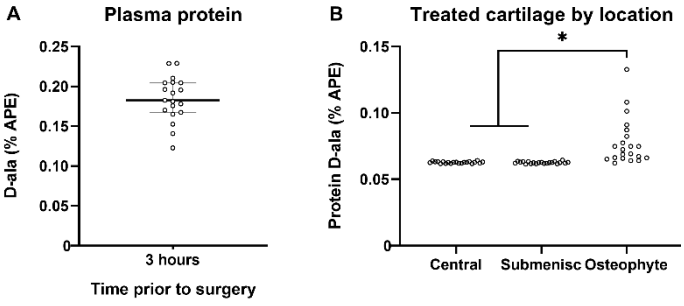

Figure 3: Protein turnover
A: D-ala levels in plasma proteins at the day of surgery. **B:** Regional D-ala in treated cartilage tissue from different locations. Values are individual data points and error bars represent median with interquartile range. D-ala: ^{2}H-alanine. APE: atomic percentage excess. * equals $P < 0.05$

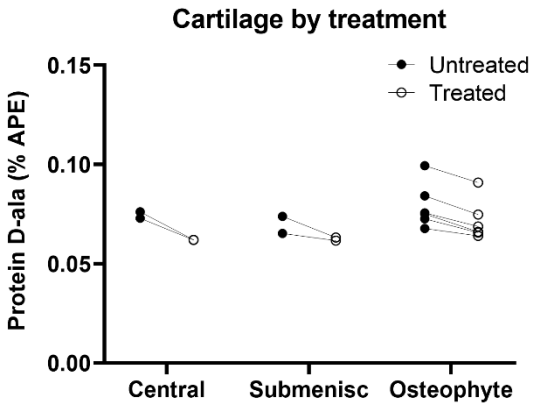

Figure 4: Effect of cartilage treatment
Cartilage samples were measured in an untreated and treated part at different regions. Values are individual data points. APE: atomic percentage excess.

DISCUSSION

Cartilage turnover of the medial tibia condyle

In this study, we wanted to determine, if the turnover of articular cartilage was different across the medial tibia condyle, as this experiences differences in load[24]. Regionally, we found no difference in collagen turnover between cartilage located centrally on the condyle or in the more peripheral region under the meniscus. Thus, despite the difference in load reported, the turnover is the same in late-stage OA. Previously, results from our lab on a similar cartilage matrix compared the life-long turnover between high-load and low-load areas and did show a positive correlation between ^{14}C levels and central location on the plateau[12]. However, the ^{14}C measurements are reflective of the accumulated tissue incorporation during the entire life. Unpublished work has determined that the correlation results from the difference in formation time due to the growth of the tibia plateau in the school years (manuscript in review).

The current gene expression results showing similarity in collagen expression but increased MMP-3 submeniscally, do mean that despite the upregulation of MMP-3, the turnover is the same under the meniscus. Currently, we did not perform any assessments of active MMP-3 or the antagonising tissue inhibitors of matrix metalloproteinases (TIMPs) enzymes, nor did we evaluate the cleaving capacity of MMP-3 on this particular matrix, so we cannot exclude that either the MMP-3 is not translated into more active MMP-3 protein, or the breakdown of the trypsin-resistant matrix is not influenced by MMP-3.

Osteophytes are a common feature in OA, and are bony outgrowths covered by fibro-cartilage[25]. The turnover of osteophytes has not been measured previously, and we are unable to compare our results with any current literature. In the present data, the small increase in deuterium incorporation found in osteophytes is somewhat unexpected, as osteophytes can develop quite rapidly especially in murine models of surgically induced OA[26]. However, the osteophyte growth is primarily due to increasing size of the bone. Therefore, the cartilage on osteophytes is possibly made continuously to support underlying bone growth by ossification of deeper zones, while the top-lining cartilage (i.e. uncalcified and thus analysed in the current setup) is kept more or less stable.

Low enrichments in the collagen matrix

In the current study, we found that the trypsin-resistant matrix of cartilage collagen contained low levels of incorporated alanine-labelled deuterium compared to plasma proteins, thus suggesting limited turnover of cartilage collagen over the past four weeks before sampling. This supports previous findings in cartilage collagen by tracing life-long ^{14}C levels[12]. On the other hand, others have shown incorporation of tritium (^{3}H) in collagen likely from the similar matrix studied currently[15]. However, in that study only five dogs were examined eight days after an intra-articular injection (one had systemic infusion) in the weeks after transection of the anterior cruciate ligament. Thus, this early phase of surgically induced OA corresponds to acute trauma rather than established post-traumatic OA, hence, the result is not comparable to ours. The current

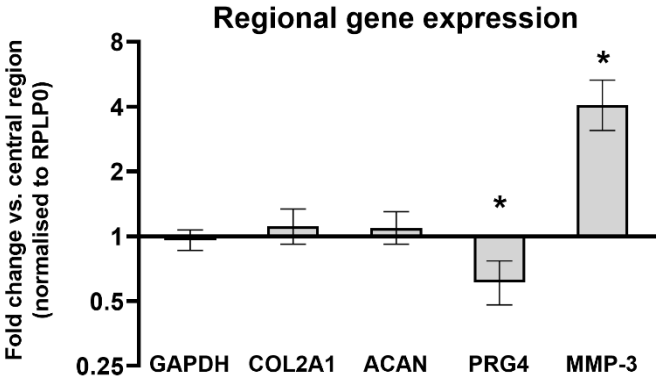

Figure 5: mRNA expression of submeniscal cartilage
The gene expression of sufficiently detected targets relative to central location normalised to RPLPo. Values are geometric means ± SEM (back transformed) shown on a logarithmic scale. * equals $P < 0.05$ compared to central location.

study was constructed to detect an incorporation of deuterium over the longest logistical period possible, and thus, minimal visits to the hospital with minimal tissue specimens were prioritized for successful recruitment of sufficient numbers of participants.

Untreated cartilage enrichment

To examine the effect of the treatment, a subset of samples of sufficient size had a part left untreated. Thus, we compared raw (untreated) and treated cartilage, and found a consistent difference with higher incorporation in untreated cartilage. The enzyme treatment would remove GAGs and proteoglycans (e.g. aggrecan), and the results show that the trypsin-resistant matrix has a lower turnover compared to untreated cartilage. By continuous infusion of stabile isotopes ($^{13}C_6$-phenylalanine) hours prior to knee replacement surgery, an FSR of 0.96%/day was found in cartilage comparable to FSR in skeletal muscle[27]. Skeletal muscle is characterized with a high synthesis rate; thus, these comparable synthesis rates, are rather unexpected. However, the synthesis of aggrecan has been demonstrated to increase with OA[28], and the high FSR in raw cartilage reported[27] could possibly thus be a result of increased aggrecan.

The limitation in this study is the inability to pin-point which specific protein is enriched in the trypsin-resistant matrix, however, assays for hydroxy-proline and GAGs on cartilage matrix using identical treatment have shown almost complete removal of GAGs and a collagen content of approximately 70% [12]. On the other hand, this means that apart from collagen, other matrix structures are similarly without any major synthesis. Investigations in the future could possibly combine mass spectrometry and proteomics to try to determine the specific proteins.

Gene expression

In support of the isotope incorporation data, we analysed gene expression of matrix components and breakdown enzymes from both the central and sub-meniscal region on the medial plateau. We found increased MMP-3 and decreased lubricin in sub-meniscal area. Unpublished data from our lab on 31 patients confirm these results (manuscript in preparation). Thus, it could seem that breakdown and reduced anabolism submeniscally exists, which could lead to increased loss of cartilage matrix. In the current study, central cartilage fibrillation was universally found, but sub-meniscal cartilage conditions varied from appearing healthy to a complete loss exposing the indurated bone. This appearance is reported by others, who similarly showed increased MMP-3 and MMP-13 in undamaged knee cartilage (corresponding to sub-meniscal) from the medial tibia plateau[29]. In femur condyles, regional differences in MMP-3 were found[30], but not by all[31,32]. We found no difference in expression of aggrecan or COL2A1 similar to many[31–33], but not all[30].

In conclusion, four weeks of deuterium labelling showed that the enrichment is similarly negligible across the medial tibia plateau and only slightly increased in osteophytes, despite regional differences in cartilage gene expression. Thus, late-stage OA cartilage seems unable to renew the stable structured collagen matrix effectively, and prevention or early intervention of OA is therefore encouraged.

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CONFLICTS OF INTEREST

The authors report no conflicts of interest.

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REFERENCES

1. Dudhia J. Aggrecan, aging and assembly in articular cartilage. *Cell Mol Life Sci*. 2005;62(19-20):2241-2256. doi:10.1007/s00018-005-5217-x

2. Heinegård D. Proteoglycans and more--from molecules to biology. *Int J Exp Pathol*. 2009;90(6):575-586. doi:10.1111/j.1365-2613.2009.00695.x

3. Eyre DR. ARTICULAR CARTILAGE COLLAGEN: AN IRREPLACEABLE FRAMEWORK? *Eur Cells Mater*. 2006;12:57-63. doi:10.22203/eCM.v012a07

4. Syx D, Tran PB, Miller RE, Malfait AM. Peripheral Mechanisms Contributing to Osteoarthritis Pain. *Curr Rheumatol Rep*. 2018;20(2). doi:10.1007/s11926-018-0716-6

5. Shane Anderson A, Loeser RF. Why is osteoarthritis an age-related disease? *Best Pract Res Clin Rheumatol*. 2010;24(1):15-26.

doi:10.1016/j.berh.2009.08.006

6. Goldring M, Otero M, Plumb D, et al. Roles of inflammatory and anabolic cytokines in cartilage metabolism: signals and multiple effectors converge upon MMP-13 regulation in osteoarthritis. *Eur Cells Mater*. 2011;21:202-220. doi:10.22203/eCM.v021a16

7. Garvican ER, Vaughan-Thomas A, Innes JF, Clegg PD. Biomarkers of cartilage turnover. Part 1: Markers of collagen degradation and synthesis. *Vet J*. 2010;185(1):36-42. doi:10.1016/j.tvjl.2010.04.011

8. Verzijl N, DeGroot J, Thorpe SR, et al. Effect of collagen turnover on the accumulation of advanced glycation end products. *J Biol Chem*. 2000;275(50):39027-39031. doi:10.1074/jbc.M006700200

9. Maroudas A, Palla G, Gilav E. Racemization of aspartic acid in human articular cartilage. *Connect Tissue Res*. 1992;28(3):161-169. http://www.ncbi.nlm.nih.gov/pubmed/1468204. Accessed August 1, 2015.

10. Canoira L, García-Martínez M-J, Llamas JF, Ortíz JE, Torres T De. Kinetics of amino acid racemization (epimerization) in the dentine of fossil and modern bear teeth. *Int J Chem Kinet*. 2003;35(11):576-591. doi:10.1002/kin.10153

11. Stabler T V, Byers SS, Zura RD, Kraus VB. Amino acid racemization reveals differential protein turnover in osteoarthritic articular and meniscal cartilages. *Arthritis Res Ther*. 2009;11(2):R34. doi:10.1186/ar2639

12. Heinemeier KM, Schjerling P, Heinemeier J, et al. Radiocarbon dating reveals minimal collagen turnover in both healthy and osteoarthritic human cartilage. *Sci Transl Med*. 2016;8(346):346ra90. doi:10.1126/scitranslmed.aad8335

13. Lippiello L, Hall D, Mankin H. Collagen synthesis in normal and osteoarthritic human cartilage. *J Clin Invest*. 1977;59(4):593-600. doi:10.1172/JCI108676

14. Hermansson M, Sawaji Y, Bolton M, et al. Proteomic Analysis of Articular Cartilage Shows Increased Type II Collagen Synthesis in Osteoarthritis and Expression of Inhibin βA (Activin A), a Regulatory Molecule for Chondrocytes. *J Biol Chem*. 2004;279(42):43514-43521. doi:10.1074/jbc.M407041200

15. Eyre DR, Mcdevitt CA, Billinghamii MEJ, Muiri H. Biosynthesis of Collagen and other Matrix Proteins by Articular Cartilage in Experimental Osteoarthrosis. *Biochem J*. 1980;188:823-837.

16. Tsuchida AI, Beekhuizen M, 't Hart MC, et al. Cytokine profiles in the joint depend on pathology, but are different between synovial fluid, cartilage tissue and cultured chondrocytes. *Arthritis Res Ther*. 2014;16(5):441. doi:10.1186/s13075-014-0441-0

17. Brook MS, Wilkinson DJ, Mitchell WK, et al. Skeletal muscle hypertrophy adaptations predominate in the early stages of resistance exercise training, matching deuterium oxide-derived measures of muscle protein synthesis and mechanistic target of rapamycin complex 1 signaling. *FASEB J*. 2015;29(11):4485-4496. doi:10.1096/fj.15-273755

18. Brook MS, Wilkinson DJ, Atherton PJ, Smith K. Recent developments in deuterium oxide tracer approaches to measure rates of substrate turnover: implications for protein, lipid, and nucleic acid research. *Curr Opin Clin Nutr Metab Care*. 2017;20(5):375-381. doi:10.1097/MCO.0000000000000392

19. Busch R, Kim Y-K, Neese R a, et al. Measurement of protein turnover rates by heavy water labeling of nonessential amino acids. *Biochim Biophys Acta*. 2006;1760(5):730-744. doi:10.1016/j.bbagen.2005.12.023

20. Altman R, Asch E, Bloch D, et al. Development of criteria for the classification and reporting of osteoarthritis: Classification of osteoarthritis of the knee. *Arthritis Rheum*. 1986;29(8):1039-1049. doi:10.1002/art.1780290816

21. Rosenblum G, Van den Steen PE, Cohen SR, et al. Direct Visualization of Protease Action on Collagen Triple Helical Structure. Buehler MJ, ed. *PLoS One*. 2010;5(6):e11043. doi:10.1371/journal.pone.0011043

22. Holm L, O'Rourke B, Ebenstein D, et al. Determination of steady-state protein breakdown rate in vivo by the disappearance of protein-bound tracer-labeled amino acids: a method applicable in humans. *Am J Physiol Endocrinol Metab*. 2013;304(8):E895-907. doi:10.1152/ajpendo.00579.2012

23. Wilkinson DJ, Franchi M V, Brook MS, et al. A validation of the application of D(2)O stable isotope tracer techniques for monitoring day-to-day changes in muscle protein subfraction synthesis in humans. *Am J Physiol Endocrinol Metab*. 2014;306(5):E571-9. doi:10.1152/ajpendo.00650.2013

24. Mononen ME, Tanska P, Isaksson H, Korhonen RK. A Novel Method to Simulate the Progression of Collagen Degeneration of Cartilage in the Knee: Data from the Osteoarthritis Initiative. *Sci Rep*. 2016;6:21415. doi:10.1038/srep21415

25. van der Kraan PM, van den Berg WB. Osteophytes: relevance and biology. *Osteoarthr Cartil*. 2007;15(3):237-244. doi:10.1016/j.joca.2006.11.006

26. Blaney Davidson EN, Vitters EL, Bennink MB, et al. Inducible chondrocyte-specific overexpression of BMP2 in young mice results in severe aggravation of osteophyte formation in experimental OA without altering cartilage damage. *Ann Rheum Dis*. 2015;74:1257-1264. doi:10.1136/annrheumdis-2013-204528

27. Smeets JSJ, Horstman AMH, Vles GF, et al. Protein synthesis rates of muscle, tendon, ligament, cartilage, and bone tissue in vivo in humans. Markofski MM, ed. *PLoS One*. 2019;14(11):e0224745. doi:10.1371/journal.pone.0224745

28. Mankin HJ, Dorfman H, Lippiello L, Zarins A. Biochemical and metabolic abnormalities in articular cartilage from osteo-arthritic human hips. II. Correlation of morphology with biochemical and metabolic data. *J Bone Joint Surg Am*. 1971;53(3):523-537. http://www.ncbi.nlm.nih.gov/pubmed/5580011. Accessed September 10, 2014.

29. Snelling S, Rout R, Davidson R, et al. A gene expression study of normal and damaged cartilage in anteromedial gonarthrosis, a phenotype of osteoarthritis. *Osteoarthr Cartil*. 2014;22(2):334-343. doi:10.1016/j.joca.2013.12.009

30. Dunn SL, Soul J, Anand S, Schwartz J-M, Boot-Handford RP, Hardingham TE. Gene expression changes in damaged osteoarthritic cartilage identify a signature of non-chondrogenic and mechanical responses. *Osteoarthr Cartil*. 2016;24(8):1431-1440. doi:10.1016/j.joca.2016.03.007

31. Yagi R, McBurney D, Laverty D, Weiner S, Horton WE. Intrajoint comparisons of gene expression patterns in human osteoarthritis suggest a change in chondrocyte phenotype. *J Orthop Res*. 2005;23(5):1128-1138. doi:10.1016/j.orthres.2004.12.016

32. Brew CJ, Clegg PD, Boot-Handford RP, Andrew JG, Hardingham T. Gene expression in human chondrocytes in late osteoarthritis is changed in both fibrillated and intact cartilage without evidence of generalised chondrocyte hypertrophy. *Ann Rheum Dis*. 2010;69(01):234-240. doi:10.1136/ard.2008.097139

33. Fukui N, Ikeda Y, Ohnuki T, et al. Regional differences in chondrocyte metabolism in osteoarthritis: A detailed analysis by laser capture microdissection. *Arthritis Rheum*. 2008;58(1):154-163. doi:10.1002/art.23175

SUPPLEMENTAL MATERIAL

Table S1: Primers

Target	Primer name	Sense	Anti-sense
RPLP0	NM_053275.3	GGAAACTCTGCATTCTCGCTTCCT	CCAGGACTCGTTTGTACCCGTTG
GAPDH	NM_002046.4	CCTCCTGCACCACCAACTGCTT	GAGGGGCCATCCACAGTCTTCT
COL2A1	NM_001844.4	ACGTGGAGATCCGGGCAGAG	CGATAACAGTCTTGCCCCACTTACC
ACAN	NM_001135.3	CTCACACAGTCGAAACAGCCACCT	TGTTCCAGCCCTCCTCACATACCTC
PRG4	NM_005807.6	TCACTGCCCAACATCAGAAAACC	GGATAAGGTCTGCCCAGAACGAG
MMP1	NM_002421.4	CGAATTTGCCGACAGAGATGAAG	GGGAAGCCAAAGGAGCTGTAGATG
MMP3	NM_002422.5	GATCCTGCTTTGTCCTTTGATGCTGT	CTGAGGGATTTGCGCCAAAAGTG
MMP13	NM_002427.4	CCTGATGACGATGTACAAGGGA	TGGCATCAAGGGATAAGGAAGGG
ADAMTS4	NM_005099.6	CCCGCTTCATCACTGACTTCCT	TGCAATGGAGCCTCTGGTTTGT
ADAMTS5	NM_007038.5	ACGCCTTGTGGAAAGGGGAGAA	AGGATCCCCAAGATCCCCAGTT

GRADUATE SCHOOL OF HEALTH AND MEDICAL SCIENCES
UNIVERSITY OF COPENHAGEN

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The declaration is for PhD students and must be completed for each conjointly authored article. Please note that if a manuscript or published paper has ten or less co-authors, all co-authors must sign the declaration of co-authorship. If it has more than ten co-authors, declarations of co-authorship from the corresponding author(s), the senior author and the principal supervisor (if relevant) are a minimum requirement.

1. Declaration by	
Name of PhD student	Adam El Mongy Jørgensen
E-mail	adam.em.jorgensen@gmail.com
Name of principal supervisor	Michael Kjær
Title of the PhD thesis	Human cartilage growth, regional turnover in vivo, and the effect of exercise in late-stage knee osteoarthritisn

2. The declaration applies to the following article	
Title of article	The regional turnover of cartilage collagen matrix in late-stage human knee osteoarthritis.
Article status	
Published ☐ Date:	Accepted for publication ☐ Date:
Manuscript submitted ☐ Date:	Manuscript not submitted ☒
If the article is published or accepted for publication, please state the name of journal, year, volume, page and DOI (if you have the information).	

3. The PhD student's contribution to the article ***(please use the scale A-F as benchmark)*** Benchmark scale of the PhD-student's contribution to the article **A.** Has essentially done all the work (> 90 %) **B.** Has done most of the work (60-90 %) **C.** Has contributed considerably (30-60 %) **D.** Has contributed (10-30 %) **E.** No or little contribution (<10 %) **F.** Not relevant	**A, B, C, D, E, F**
1. Formulation/identification of the scientific problem	C
2. Development of the key methods	F
3. Planning of the experiments and methodology design and development	B
4. Conducting the experimental work/clinical studies/data collection/obtaining access to data	A
5. Conducting the analysis of data	C
6. Interpretation of the results	C
7. Writing of the first draft of the manuscript	A
8. Finalisation of the manuscript and submission	A
Provide a short description of the PhD student's specific contribution to the article.[i] The PhD student wrote the protocol, obtained ethical permission, and set-up a collaboration with the clinical staff of relevant University Hospital departments of orthopaedic surgery. Further, the PhD student recruited participants, coordinated and facilitated the intervention, performed the sampling of biopsies from the cartilage waste tissue obtained from surgery, and prepared the samples for isotope analysis. Finally, the PhD student performed statistics with co-authors, made all the figures, wrote the initial manuscript, and incorporated comments and feedback from co-authors into the final manuscript.	

4. Material from another thesis / dissertation[ii]	
Does the article contain work which has also formed part of another thesis, e.g. master's thesis, PhD thesis or doctoral dissertation (the PhD student's or another person's)?	Yes: ☐ No: ☒
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If the article is part of another author's academic degree, please describe the PhD student´s and the author´s contributions to the article so that the individual contributions are clearly distinguishable from one another.	N/A

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10.				

6. Signature of the principal supervisor

I solemnly declare that the information provided in this declaration is accurate to the best of my knowledge.
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Principal supervisor: Michael Kjær

7. Signature of the PhD student

I solemnly declare that the information provided in this declaration is accurate to the best of my knowledge.
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PhD student: Adam El Mongy Jørgensen 11/8-20

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[iii] If more signatures are needed please add an extra sheet.

Acute loading has minor influence on human articular cartilage in late-stage knee osteoarthritis: A randomised controlled trial

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ABSTRACT **Background:** Osteoarthritis (OA) remains a great clinical challenge. Although regular physical activity has shown positive effects upon pain and functional outcomes in OA patients, it is unknown how physical activity influences cartilage dynamics. Thus, we aim to determine the effect of acute exercise upon human OA cartilage.

Methods: A single-blind randomised controlled trial with a parallel two-arm design containing usual care or a single resistance exercise bout (one-legged leg-press) 3.5 hours prior to scheduled joint replacement surgery due to primary knee OA. Cartilage from the medial tibia condyle was sampled a) centrally, b) under the meniscus, and c) from peripheral osteophytes. Samples were analysed for gene expression using real-time reverse transcriptase polymerase chain reaction.

Results: Of 32 patients randomised, 31 completed the intervention: mean age 69±7.5 years (SD), 58% female, BMI 29.4±4.4 kg/m² (SD). Acute exercise increased TGF-β1 gene expression 1.23-fold (P = 0.048) with no effect on other targets. Regionally, submeniscal area showed increased MMP-3, MMP-13, IGF-1Ea, and CTGF expression, and decreased expression of lubricin and COMP compared to the central condyle. Further, osteophytes showed increased expression of MMP-1, MMP-13, IGF-1Ea, and TGF-β3, and decreased expression of aggrecan, COMP, and FGF-2 compared to articular cartilage.

Conclusion: In OA, the articular cartilage surface contains regional differences in gene expression with an unfavourable environment under the meniscus. A single resistance exercise bout has minor influence on cartilage.

Keywords: Physical activity, resistance exercise, messenger RNA, osteophyte, regional

INTRODUCTION

Osteoarthritis (OA) is a painful and debilitating disease of the joint [1], which occurs with an increasing frequency with ageing [2]. OA is ultimately treated by joint replacement surgery – an effective treatment, however, functional outcomes vary and the lifespan of the prostheses itself is limited [3]. Many non-surgical treatments are recommended, including pain-reducing medication, weight loss, and exercise [4], and systematic reviews show that exercise is clinically beneficial [5] and safe for the cartilage [6].

As sampling from human cartilage is highly invasive and damaging, measurements of cartilage biomarkers in blood, urine, and synovial fluid (SF) in connection to exercise have been made, with months of exercise generally providing reduced markers of cartilage breakdown and inflammation in OA [7]. However, when sampling systemically, it is very difficult to make firm conclusions on the cartilage in a single joint, and a biomarker specifically for cartilage has yet to be found.

Studies subjecting human chondrocytes or cartilage explants to mechanical load have shown the existence of mechanically regulated genes within groups of matrix proteins, cytokines, growth factors, and degrading enzymes [8]. However, mixed results have been found as the setup varies regarding tissue preparation, as well as for type and dose of load, which further complicates the extrapolation to *in vivo* conditions [8]. Thus, despite the comprehensive knowledge, the effect of exercise on human cartilage itself is unknown. Therefore, we aimed to examine the effect of a single bout of resistance exercise upon gene expression in human OA cartilage from the knee. As the clinical effect of exercise is well

described [9], we hypothesise that exercise will provide a beneficial anti-catabolic and/or anti-inflammatory cellular response.

METHODS

Trial design

This was a single day two-arm randomised clinical trial of resistance exercise intervention compared to no exercise (usual care) in patients with OA of the knee scheduled for joint replacement surgery. Outcomes included gene expression changes. The study was performed on Copenhagen University Hospital of Bispebjerg in Denmark. Participants gave written informed content and the study was approved by the Ethical Committee of the Capital Region of Denmark (H-17015563) in accordance with the Helsinki Declaration of 1975/83. The trial was registered on ClinicalTrials.gov (NCT03410745).

Participants

Patients were recruited from the outpatient clinic at the University Hospital orthopaedic department. Patients with OA of the knee as classified by the American College of Rheumatology [10] scheduled for a knee replacement surgery by an orthopaedic surgeon were considered for participation. Patients were eligible if they were 50–90 years old, had primary OA, were able to perform physical exercise, had a body mass index (BMI) within 18.5–40 kg/m^2, and were non-smoker.

Exclusion criteria included other diseases of the joint (rheumatoid arthritis (RA), gout, and psoriasis arthritis), inflammatory diseases, diabetes or cancer, previous knee surgery or recent trauma to the knee, or participation in regular strenuous exercise, or required an interpreter. Further, the use of non-steroid anti-inflammatory drugs (NSAIDs), oral corticoid-steroids or intraarticular injection within three months, disease modifying anti-rheumatic drugs (DMARDs), chemotherapy, or other immuno-suppressive medicine were also considered ineligible.

Intervention

The exercise intervention was supervised by a medical doctor and began by a light warm-up on an ergometer bike for 5–10 minutes. In a leg-press machine (Techno Gym, Cesena, Italy), participants were seated upright with the hip bended 90°. To ensure load on the knee of interest, one-legged exercise was performed with the working leg extending from 100° to near fully stretched against a vertical plate, and the resting leg positioned on a horizontal plate. Due to safety concerns of the overnight fasted participants with knee pain, we performed a 5 repetition maximum (RM) test as an indirect measure of maximum strength to calculate the corresponding 1 RM[11]. Three sets of eight repetitions at 70% 1 RM and three sets of four repetitions at 80% 1 RM were performed at controlled speed.

Randomisation

Participants were randomly allocated 1:1 to one of the two groups using the minimisation software MinimPy version 0.3 (Python Software Foundation, Beaverton, OR, USA) [12], stratified by sex, age (<70 or ≥70 years), and BMI (18.5–25, 25.1–30, or 30.1–40 kg/m^2). Due to the nature of the intervention, the participants were unblinded, however, to the best of our knowledge, the outcome measurements cannot be influenced willingly. Surgeons were unaware of the allocated group during surgery, and messenger ribonucleic acid (mRNA) data were obtained blinded.

Tissue collection

During knee replacement surgery, the leftover tibia plateau was collected and immediately transferred to the laboratory on ice. From the medial tibia condyle, several full-thickness cartilage biopsies were sampled centrally and under the meniscus. From osteophytes (if present), slices were sampled using a scalpel. By cutting with a scalpel, the subchondral bone was removed from biopsies before the cartilage was snap-frozen in liquid nitrogen and kept on −80°C until further analysis.

Gene expression analysis

Cartilage biopsies were cut in 10 µm slices at −20°C. The slices were homogenised in TriReagent (Molecular Research Center, Cincinnati, OH, USA) using 2.3 mm stainless steel beads in a homogenising tube (BioSpec Products Inc., Bartlesville, OK, USA) and shaken (FastPrep, FP Biomedicals, Illkirch, France). Bromo-chloropropane (Sigma, Darmstadt, Germany) was added to isolate RNA containing aqueous phase, where a poly-acryl carrier and isopropanol were added to precipitate the mRNA. The pellet was washed with 75% ethanol, dissolved in water, transferred to spin columns (Quiagen, Hilden, Germany) for further washing, and then finally eluted with water. The RNA concentration was determined by the RiboGreen assay (Molecular Probes Inc., Eugene, OR, USA) as per manufacture's protocol, which uses a fluorescent nucleic acid stain for quantitating RNA from a standard curve. The extracted

mRNA was then converted to 50 ng cDNA using poly-dT (Invitrogen, Nærum, Denmark) with the OmniScript Kit (Qiagen, Hilden, Germany) as per manufacture's protocol. Real-time RT-PCR (Stratagene, La Jolla, CA, USA) with specific primers (table S1) were performed using Ct values from a standard curve of DNA oligos (Ultramer, Integrated DNA Technologies Inc., Leuven, Belgium) with a DNA sequence corresponding to the expected PCR product. The specificity of the PCR products was confirmed by melt curves of unknown samples and known oligos. Expressed values were normalised to internal control of large ribosomal protein Po (RPLPo), which was then validated using glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Statistics

Statistical analyses were performed using SigmaPlot version 13.0 (Systat Software Inc., San Jose, CA, USA) and figures were made in GraphPad Prism version 8.4.1 (GraphPad Software, San Diego, CA, USA). Baseline results were compared using Mann-Whitney test. Effects of exercise intervention and regional location were analysed using repeated-measures two-way analysis of variance (ANOVA) with Tukey's multiple comparisons test post hoc on logarithmic values. Statistical significance was defined as *P*-values < 0.05 for analyses.

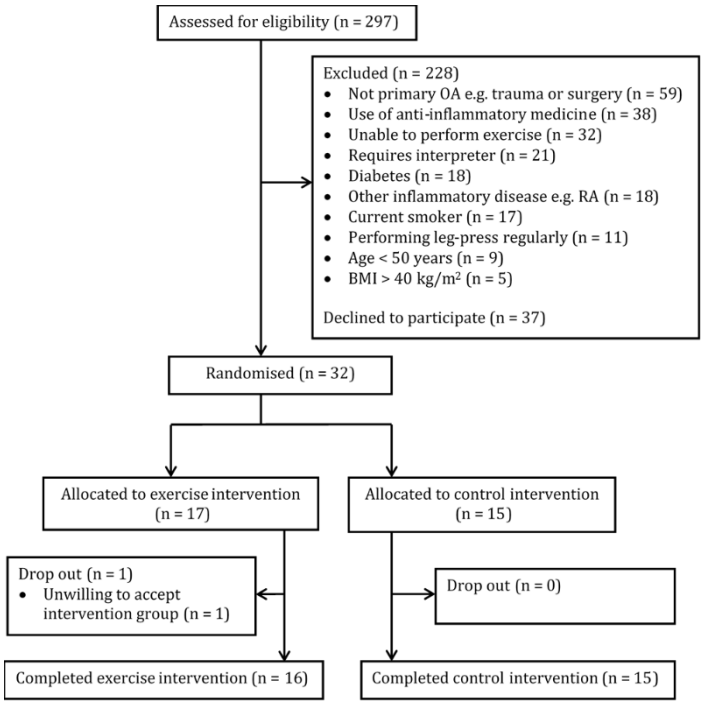

Figure 1: CONSORT flow chart

The flow of patients/participants through the current study.

Table 1: Participants' characteristics

Parameter	Control (n = 15)	Exercise (n = 16)
Age (years)	67.8 ± 8.0	69.5 ± 7.2
Body mass index (kg/m2)	29.7 ± 3.2	29.2 ± 5.4
Sex (percentage female)	67%	50%
Kellgren-Lawrence grade (0-4)	3.6 ± 0.5	3.4 ± 0.5
5 repetition maximum (kg)	N/A	112.5 ± 31.9
Time from exercise to tissue freezing (h:mm)	N/A	3:24 ± 0:37

N/A: Not applicable. Values are means ± SD

RESULTS

The 32 participants were recruited between May 2018 and September 2019 (Fig. 1), with similar baseline characteristics in the two groups (table 1). During the exercise intervention, no harms were found.

We extracted low amounts of RNA, but found no difference in GAPDH, thus validating the use of RPLPo for normalisation (Fig. 2A-B).

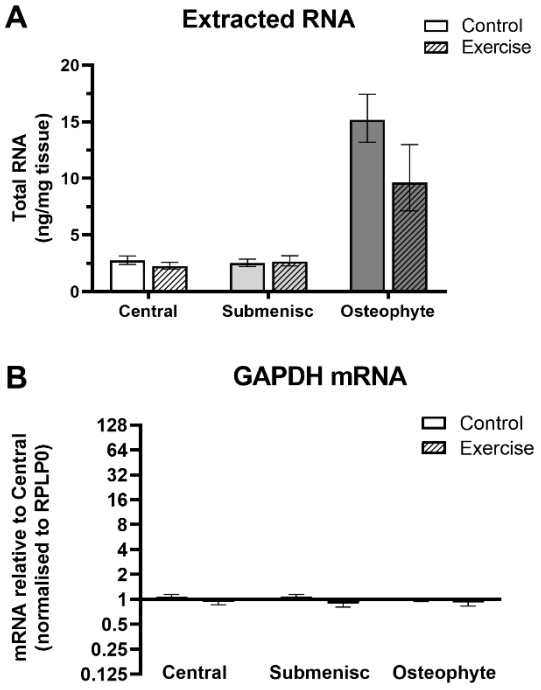

Figure 2: RNA extraction and GAPDH expression

A: Total amount of extracted RNA. **B:** The gene expression of GAPDH relative to central location. Values are geometric means ± SEM (back transformed).

Four samples were of insufficient quality (two in each group), and in total, 23 genes were assessed of which 13 were detected sufficiently for analyses (Fig. 3 and table S2).

The effect of exercise was only significant for tissue growth factor beta 1 (TGF-β1) with a 1.23-fold increase, (P = 0.048) (Fig. 3B). When compared to the central condyle area, we found significantly increased expression of matrix metalloproteinase 3 (MMP-3), MMP-13, insulin-like growth factor 1 (IGF-1Ea), and connective tissue growth factor (CTGF), and further, we found decreased expression of lubricin (PRG4) and cartilage oligomeric protein (COMP) in the submeniscal region (Fig. 3A–B).

Compared to articular cartilage, osteophytes showed increased expression of MMP-1, MMP-13, IGF-1Ea, and TGF-β3 with decreased expression of aggrecan (ACAN), COMP, and fibroblast growth factor 2 (FGF-2) (Fig. 3A–B).

DISCUSSION

Acute exercise has minor influence on chondrocyte gene expression

We conducted a randomised controlled trial in order to examine the effect of exercise on gene expression from human OA chondrocytes from the tibia plateau and only found TGF-β1 expression to be slightly increased by exercise 3.5 hours after a single bout of resistance exercise. However, this is the first study to examine the effect of exercise on gene expression in humans *in vivo*, and thus we are unable to compare our results directly against the literature. Consequently, it cannot be ruled out that in humans with OA, chondrocyte gene expression is in fact not regulated in a detectable manner by this single bout of exercise setup within the given timeframe.

In line with our findings, *in vitro* loading of aged but healthy bovine cartilage [13] and of human OA synovial fibroblasts [14] caused increased TGF-β1 expression. However, as TGF-β exists in a latent complex bound to

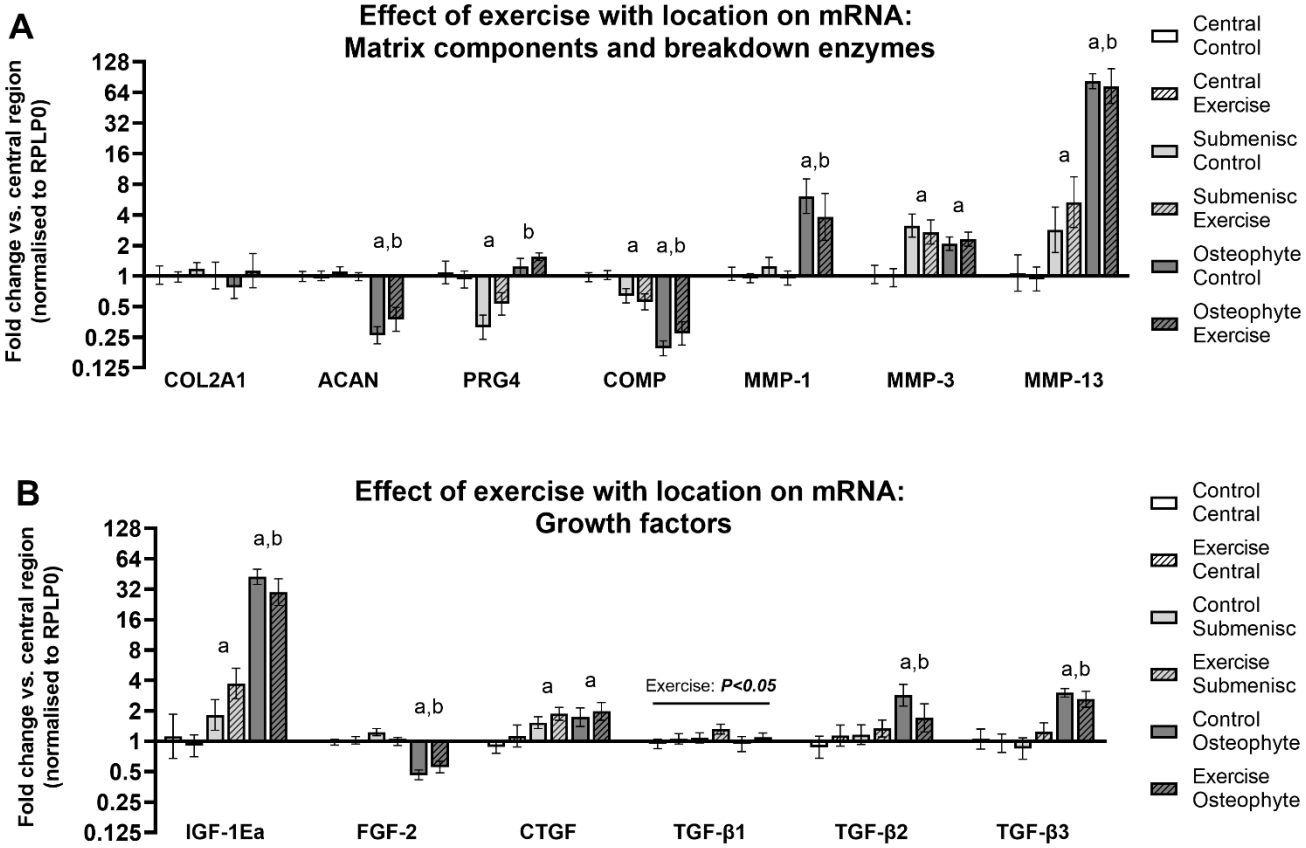

Figure 3: Effect of exercise on gene expression

The fold change of every region is shown for sufficiently detected targets relative to central region normalised to RPLPo. **A:** Matrix components and breakdown enzymes. **B:** Growth factors. Regions are shown in white (central), light grey (submenisc), and dark grey (osteophyte) colours and exercise by striped fill pattern on top. Values are geometric means ± back transformed SEM. **a** and **b** equals $P < 0.05$ of main effect vs. central or submeniscal location respectively.

CTGF in the pericellular matrix, reaching its receptor when cartilage is loaded [15], the effect of a slight increase in gene expression in uncertain. Further, TGF-β seems to be vital during development and normal cartilage maintenance, but potentially destructive in OA on the other hand [16], although an anti-inflammatory effect on synovial fibroblasts has been found [17]. Thus, our results suggest that TGF-β1 expression can respond to exercise in human OA cartilage as well, albeit conclusions on its effect is speculative.

OA is characterised by increased catabolism of the cartilage where GAGs are removed, thereby exposing the underlying collagen fibres for digestion primarily by MMP-13 [18]. However, increased synthesis of matrix components is also seen in OA cartilage [19], and this failed repair attempt has been compared to a chronic ulcer, where the matrix breakdown products activate inflammation by toll like receptors (TLR) [20]. In humans performing acute exercise, only biomarker studies exist mainly measuring in serum [21], while e.g. synovial fluid (SF) sampling in patients with OA is sparse [22].

Decreased SF level of COMP has earlier been found after acute exercise [23], however, the baseline measurement were made three months earlier, and thus, an effect of time cannot be ruled out. Although COMP has been widely examined for breakdown of cartilage, COMP is also expressed in synovial cells [24], which somewhat complicates the measurements on SF. In our present study, we did not detect mRNA from interleukins (ILs) similarly to others [25], which raises the question of whether cells from other regions (i.e. synovial lining or subchondral bone) could in fact be the population responsible for IL production. Another possibility is different tissue preparation, as a study found marked increases in IL expression *in vitro* compared to *in vivo* [26]. In patients with moderate OA (Kellgren-Lawrence 2–3), increased SF and perisynovial IL-10 was found in the recovery period after acute exercise, however, IL-10 levels were not measured before exercise [27]. In our setup with direct visualisation of the joint capsule during surgery, too few SF samples were collectable to perform feasible SF analysis, and a sample from the synovial membrane were not ethically possible. Thus, further conclusions on IL-10 with exercise remains speculative.

In healthy human chondrocytes that are exposed to load, MMP-1 is either unchanged [28], or decreases together with MMP-3 and -13 due to increasing IL-4 [29–31]. However, in OA there was no change in MMP-1 or -3 [28,29], similar to our data. Further, in the present study we barely detected MMP-1, consistent with one study [32] but differently from others [33,34]. Thus, our human exercise data are in line with *in vitro* studies, and MMPs does not seem to be influenced within our setup. Finally, ADAMTS-4 was undetected and ADAMTS-5 was found in too low amount contrary to others [32,34]. From our data, additional conclusions on ADAMTS are speculative.

In the current study, no change in lubricin gene expression of chondrocytes with exercise was found. Synoviocytes and superficial zone chondrocytes expresses lubricin, which reduces the sheer stress to the surface and protects against chondrocyte apoptosis [35]. In an rat OA model, lubricin injections inhibited TLR [36], thus providing cartilage protection. In healthy rodents, low and moderate intensity running increased lubricin gene expression after 4-8 weeks [37,38]. A single study has measured the loading effect *in vivo* in rats with a damaged joint, mimicking early post-traumatic OA. Here, an ACL lesion reduced lubricin expression, which was then further reduced by forced running [39]. Thus, in OA, exercise-induced lubricin secretion from chondrocytes are uncertain at best, and from a study applying *in vivo* stretching of synovial fibroblasts, increased lubricin gene expression was found from healthy but not OA donors [14].

With acute exercise, we did not find any effect upon aggrecan expression. Similarly, in OA chondrocytes, loading did not change aggrecan expression, which was observed by an increase in healthy chondrocytes [28]. Using delayed gadolinium-enhanced magnetic resonance imaging of cartilage (dGEMRIC) as a surrogate marker for GAG content, long-term exercise provided no change in tibiofemoral cartilage with mild OA [40–42]. Thus, in OA, an exercise-induced increase in GAG content seems unattainable. Taken together, the beneficial effect of exercise on healthy cartilage could not be verified in OA cartilage in the current setup, and the clinical improvement is suggested to be from an extra chondral origin e.g. extra articular effects on muscles.

Both MMPs and a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) 4 and 5 cleaves aggrecan in human chondrocytes [43–45], and excessive load has been shown to decrease cartilage GAG content in healthy animals [46,47]. Currently, exercise did not lead to increased expression of breakdown enzymes nor cytokines, and consequently, in patients with late-stage OA, the performed exercise intervention appears safe for cartilage.

One limitation of the study is that only 23% of the patients screened were eligible for participation, which limit the generalisability of the study. However, the comprehensive criteria optimised the possibility of identifying an effect of exercise, and the trial participants are believed to represent the most likely to respond to exercise. Hence, an effect in e.g. post-traumatic OA cannot be excluded. Another limitation is the intervention itself. Our intervention could show an effect at later than the 3.5-hours timeframe, or it could be inadequate as a stimulus, as the participants did not perform an all-out resistance exercise bout. However, the performed exercise uses moderate to hard load, and thus, the cartilage should be loaded sufficiently. Further, despite our participants being overnight fasted and untrained, all completed the protocol without harms, similarly to others [48], thus making the intervention clinically possible and applicable. Finally, the effect of a single resistance exercise bout might be undetectable, as months of exercise that tended to show a beneficial response on cartilage biomarkers [7]. This could be speculated to result from an increased synthesis, although reduced breakdown cannot be ruled out or determined.

Regional differences in gene expression

As the submeniscal area on the medial plateau experience higher load than central [8,49], we sampled from both regions. Our data showed increased MMP-3, MMP-13, IGF-1Ea, and CTGF, and decreased lubricin and COMP in submeniscal area. Unpublished observations from our lab shows similar regional differences for MMP-3 and lubricin, while the other targets were unanalysed. Thus, it appears that breakdown, fibrosis, and reduced anabolism dominates submeniscally. In this study, cartilage fibrillation was universally found centrally, whereas submeniscal cartilage conditions varied from appearing healthy to a complete loss. This appearance is reported by others, who similarly to us showed increased MMP-3, MMP-13, and IGF-1Ea and decreased COMP expression in undamaged cartilage (corresponding to submeniscial) from the medial tibia plateau [33]. From femur condyles, results varies: Some too found differences in MMP-3 [25], and MMP-13 [50], while others did not [51,52]. We found no difference in expression of aggrecan or COL2A1 similar to many [51–53], although decreased aggrecan has been found centrally [25].

Osteophytes are bony outgrowths covered by fibro-cartilage [54], and compared to articular cartilage, we found decreased aggrecan, COMP, and FGF-2 and increased MMP-1, MMP-13, TGF-β3, and IGF-1Ea compatible with osteophyte growth and ossification capability. Very limited work has been done in humans on chondrocyte gene expression from osteophytes, but similar increases in TGF-β and unchanged FGF-2 have been found [55], and while IGF-1 was found expressed in osteophytes, the level was not compared to articular cartilage [56]. Thus, osteophyte gene expression differs from articular cartilage, and the pattern conforms to osteophyte growth, remodelling, and ossification capability supporting the underlying bony growth. Hence, care should be made during OA cartilage tissue sampling of an entire joint surface for RNA analysis.

In conclusion, by performing a randomised controlled trial in patients with advanced OA, we found that acute exercise has minor effect on gene expression after 3.5 hours. Thus, within the cartilage tissue exercise appears safe albeit not beneficial. Further, on the medial tibia condyle surface, differences in gene expression were found between central and submeniscal areas, and osteophytes.

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CONFLICTS OF INTEREST

The authors report no conflicts of interest.

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REFERENCES

1. Syx D, Tran PB, Miller RE, Malfait AM. Peripheral Mechanisms Contributing to Osteoarthritis Pain. *Curr Rheumatol Rep*. 2018;20(2). doi:10.1007/s11926-018-0716-6

2. Shane Anderson A, Loeser RF. Why is osteoarthritis an age-related disease? *Best Pract Res Clin Rheumatol*. 2010;24(1):15-26.

doi:10.1016/j.berh.2009.08.006

3. Glyn-Jones S, Palmer AJR, Agricola R, Price AJ, Vincent TL, Weinans H, et al. Osteoarthritis. *Lancet*. 2015;386(9991):376-387. doi:10.1016/S0140-6736(14)60802-3

4. Kolasinski SL, Neogi T, Hochberg MC, Oatis C, Guyatt G, Block J, et al. 2019 American College of Rheumatology/Arthritis Foundation Guideline for the Management of Osteoarthritis of the Hand, Hip, and Knee. *Arthritis Care Res (Hoboken)*. 2020;72(2):149-162. doi:10.1002/acr.24131

5. Fransen M, McConnell S, Harmer AR, Van der Esch M, Simic M, Bennell KL. Exercise for osteoarthritis of the knee. Fransen M, ed. *Cochrane Database Syst Rev*. January 2015. doi:10.1002/14651858.CD004376.pub3

6. Bricca A. Exercise does not "wear down my knee": systematic reviews and meta-analyses. *Br J Sports Med*. 2018;52(24):1591-1592. doi:10.1136/bjsports-2018-099705

7. Bricca A, Struglics A, Larsson S, Steultjens M, Juhl CB, Roos EM. Impact of exercise therapy on molecular biomarkers related to cartilage and inflammation in people at risk of, or with established, knee osteoarthritis: a systematic review and meta-analysis of randomized controlled trials. *Arthritis Care Res (Hoboken)*. October 2018. doi:10.1002/acr.23786

8. Wong M, Carter D. Articular cartilage functional histomorphology and mechanobiology: a research perspective. *Bone*. 2003;33(1):1-13. doi:10.1016/S8756-3282(03)00083-8

9. Kraus VB, Sprow K, Powell KE, Buchner D, Bloodgood B, Piercy K, et al. Effects of Physical Activity in Knee and Hip Osteoarthritis: A Systematic Umbrella Review. *Med Sci Sports Exerc*. 2019;51(6):1324-1339. doi:10.1249/MSS.0000000000001944

10. Altman R, Asch E, Bloch D, Bole G, Borenstein D, Brandt K, et al. Development of criteria for the classification and reporting of osteoarthritis: Classification of osteoarthritis of the knee. *Arthritis Rheum*. 1986;29(8):1039-1049. doi:10.1002/art.1780290816

11. Brzycki M. Strength Testing—Predicting a One-Rep Max from Reps-to-Fatigue. *J Phys Educ Recreat Danc*. 1993;64(1):88-90. doi:10.1080/07303084.1993.10606684

12. Saghaei M, Saghaei S. Implementation of an open-source customizable minimization program for allocation of patients to parallel groups in clinical trials. *J Biomed Sci Eng*. 2011;04(11):734-739. doi:10.4236/jbise.2011.411090

13. Madej W, van Caam A, Blaney Davidson EN, Hannink G, Buma P, van der Kraan PM. Ageing is associated with reduction of mechanically-induced activation of Smad2/3P signaling in articular cartilage. *Osteoarthr Cartil*. 2016;24(1):146-157. doi:10.1016/j.joca.2015.07.018

14. Jamal J, Roebuck MM, Lee S-Y, Frostick SP, Abbas AA, Merican AM, et al. Modulation of the mechanical responses of synovial fibroblasts by osteoarthritis-associated inflammatory stressors. *Int J Biochem Cell Biol*. 2020;126:105800. doi:10.1016/j.biocel.2020.105800

15. Tang X, Muhammad H, McLean C, Miotla-Zarebska J, Fleming J, Didangelos A, et al. Connective tissue growth factor contributes to joint homeostasis and osteoarthritis severity by controlling the matrix sequestration and activation of latent TGFβ. *Ann Rheum Dis*. 2018;77(9):1372-1380. doi:10.1136/annrheumdis-2018-212964

16. van der Kraan PM. The changing role of TGFβ in healthy, ageing and osteoarthritic joints. *Nat Rev Rheumatol*. 2017;13(3):155-163. doi:10.1038/nrrheum.2016.219

17. Kuo S-J, Liu S-C, Huang Y-L, Tsai C-H, Fong Y-C, Hsu H-C, et al. TGF-β1 enhances FOXO3 expression in human synovial fibroblasts by inhibiting miR-92a through AMPK and p38 pathways. *Aging (Albany NY)*. 2019;11(12):4075-4089. doi:10.18632/aging.102038

18. Goldring M, Otero M, Plumb D, Dragomir C, Favero M, EI Hachem K, et al. Roles of inflammatory and anabolic cytokines in cartilage metabolism: signals and multiple effectors converge upon MMP-13 regulation in osteoarthritis. *Eur Cells Mater*. 2011;21:202-220. doi:10.22203/eCM.v021a16

19. Sandy JD, Chan DD, Trevino RL, Wimmer MA, Plaas A. Human genome-wide expression analysis reorients the study of inflammatory mediators and biomechanics in osteoarthritis. *Osteoarthr Cartil*. 2015;23(11):1939-1945. doi:10.1016/j.joca.2015.03.027

20. Scanzello CR, Plaas A, Crow MK. Innate immune system activation in osteoarthritis: is osteoarthritis a chronic wound? *Curr Opin Rheumatol*. 2008;20(5):565-572. doi:10.1097/BOR.0b013e32830aba34

21. Roberts HM, Law RJ, Thom JM. The time course and mechanisms of change in biomarkers of joint metabolism in response to acute exercise and chronic training in physiologic and pathological conditions. *Eur J Appl Physiol*. 2019;119(11-12):2401-2420. doi:10.1007/s00421-019-04232-4

22. Mazor M, Best TM, Cesaro A, Lespessailles E, Toumi H. Osteoarthritis biomarker responses and cartilage adaptation to exercise: A review of animal and human models. *Scand J Med Sci Sports*. 2019;29(8):sms.13435. doi:10.1111/sms.13435

23. Helmark IC, Petersen MCH, Christensen HE, Kjaer M, Langberg H. Moderate loading of the human osteoarthritic knee joint leads to lowering of intraarticular cartilage oligomeric matrix protein. *Rheumatol Int*. 2012;32(4):1009-1014. doi:10.1007/s00296-010-1716-7

24. Tseng S, Reddi AH, Di Cesare PE. Cartilage Oligomeric Matrix Protein (COMP): A Biomarker of Arthritis. *Biomark Insights*. 2009;4(4):33-44. doi:10.4137/bmi.s645

25. Dunn SL, Soul J, Anand S, Schwartz J-M, Boot-Handford RP, Hardingham TE. Gene expression changes in damaged osteoarthritic cartilage identify a signature of non-chondrogenic and mechanical responses. *Osteoarthr Cartil*. 2016;24(8):1431-1440. doi:10.1016/j.joca.2016.03.007

26. Tsuchida AI, Beekhuizen M, 't Hart MC, Radstake TRDJ, Dhert WJA, Saris DBF, et al. Cytokine profiles in the joint depend on pathology, but are different between synovial fluid, cartilage tissue and cultured chondrocytes. *Arthritis Res Ther*. 2014;16(5):441. doi:10.1186/s13075-014-0441-0

27. Helmark IC, Mikkelsen UR, Børglum J, Rothe A, Petersen MCH, Andersen O, et al. Exercise increases interleukin-10 levels both intraarticularly and peri-synovially in patients with knee osteoarthritis: a randomized controlled trial. *Arthritis Res Ther*. 2010;12(4):R126. doi:10.1186/ar3064

28. Millward-Sadler SJ, Wright MO, Davies LW, Nuki G, Salter DM. Mechanotransduction via integrins and interleukin-4 results in altered aggrecan and matrix metalloproteinase 3 gene expression in normal, but not osteoarthritic, human articular chondrocytes. *Arthritis Rheum*. 2000;43(9):2091-2099. doi:10.1002/1529-0131(200009)43:9<2091::AID-ANR21>3.0.CO;2-C

29. Salter DM, Millward-Sadler SJ, Nuki G, Wright MO. Differential responses of chondrocytes from normal and osteoarthritic human articular cartilage to mechanical stimulation. *Biorheology*. 2002;39:97-108.

30. Leong DJ, Li YH, Gu XI, Sun L, Zhou Z, Nasser P, et al. Physiological loading of joints prevents cartilage degradation through CITED2. *FASEB J*. 2011;25(1):182-191. doi:10.1096/fj.10-164277

31. He Z, Leong DJ, Xu L, Hardin JA, Majeska RJ, Schaffler MB, et al. CITED2 mediates the cross-talk between mechanical loading and IL-4 to promote chondroprotection. *Ann N Y Acad Sci*. 2019;1442(1):128-137. doi:10.1111/nyas.14021

32. Bau B, Gebhard PM, Haag J, Knorr T, Bartnik E, Aigner T. Relative messenger RNA expression profiling of collagenases and aggrecanases in human articular chondrocytes in vivo and in vitro. *Arthritis Rheum*. 2002;46(10):2648-2657. doi:10.1002/art.10531

33. Snelling S, Rout R, Davidson R, Clark I, Carr A, Hulley PA, et al. A gene expression study of normal and damaged cartilage in anteromedial gonarthrosis, a phenotype of osteoarthritis. *Osteoarthr Cartil*. 2014;22(2):334-343. doi:10.1016/j.joca.2013.12.009

34. Jessberger S, Högger P, Genest F, Salter DM, Seefried L. Cellular pharmacodynamic effects of Pycnogenol® in patients with severe osteoarthritis: a randomized controlled pilot study. *BMC Complement Altern Med*. 2017;17(1):537. doi:10.1186/s12906-017-2044-1

35. Waller KA, Zhang LX, Elsaid KA, Fleming BC, Warman ML, Jay GD. Role of lubricin and boundary lubrication in the prevention of chondrocyte apoptosis. *Proc Natl Acad Sci*. 2013;110(15):5852-5857. doi:10.1073/pnas.1219289110

36. Iqbal SM, Leonard C, C Regmi S, De Rantere D, Tailor P, Ren G, et al. Lubricin/Proteoglycan 4 binds to and regulates the activity of Toll-Like Receptors In Vitro. *Sci Rep*. 2016;6:18910. doi:10.1038/srep18910

37. Ni G-X, Lei L, Zhou Y-Z, Su J, Schumacher B, Lindley K, et al. Intensity-dependent effect of treadmill running on lubricin metabolism of rat articular cartilage. *Arthritis Res Ther*. 2012;14(6):R256. doi:10.1186/ar4101

38. Ogawa H, Kozhemyakina E, Hung H-H, Grodzinsky AJ, Lassar AB. Mechanical motion promotes expression of Prg4 in articular cartilage via multiple CREB-dependent, fluid flow shear stress-induced signaling pathways. *Genes Dev*. 2014;28(2):127-139. doi:10.1101/gad.231969.113

39. Elsaid KA, Zhang L, Waller K, Tofte J, Teeple E, Fleming BC, et al. The impact of forced joint exercise on lubricin biosynthesis from articular cartilage following ACL transection and intra-articular lubricin's effect in exercised joints following ACL transection. *Osteoarthr Cartil*. 2012;20(8):940-948. doi:10.1016/j.joca.2012.04.021

40. Multanen J, Nieminen MT, Häkkinen A, Kujala UM, Jämsä T, Kautiainen H, et al. Effects of high-impact training on bone and articular cartilage: 12-month randomized controlled quantitative MRI study. *J Bone Miner Res*. 2014;29(1):192-201. doi:10.1002/jbmr.2015

41. Munukka M, Waller B, Rantalainen T, Häkkinen A,

Nieminen MT, Lammentausta E, et al. Efficacy of progressive aquatic resistance training for tibiofemoral cartilage in postmenopausal women with mild knee osteoarthritis: a randomised controlled trial. *Osteoarthr Cartil*. 2016;24(10):1708-1717. doi:10.1016/j.joca.2016.05.007

42. Multanen J, Rantalainen T, Kautiainen H, Ahola R, Jämsä T, Nieminen MT, et al. Effect of progressive high-impact exercise on femoral neck structural strength in postmenopausal women with mild knee osteoarthritis: a 12-month RCT. *Osteoporos Int*. 2017;28(4):1323-1333. doi:10.1007/s00198-016-3875-1

43. Song R-H, D. Tortorella M, Malfait A-M, Alston JT, Yang Z, Arner EC, et al. Aggrecan degradation in human articular cartilage explants is mediated by both ADAMTS-4 and ADAMTS-5. *Arthritis Rheum*. 2007;56(2):575-585. doi:10.1002/art.22334

44. Dudhia J, Davidson CM, Wells TM, Vynios DH, Hardingham TE, Bayliss MT. Age-related changes in the content of the C-terminal region of aggrecan in human articular cartilage. *Biochem J*. 1996;313 (Pt 3:933-940.

45. Struglics A, Hansson M. MMP proteolysis of the human extracellular matrix protein aggrecan is mainly a process of normal turnover. *Biochem J*. 2012;446(2):213-223. doi:10.1042/BJ20120274

46. Arokoski JP, Jurvelin JS, Väätäinen U, Helminen HJ. Normal and pathological adaptations of articular cartilage to joint loading. *Scand J Med Sci Sports*. 2000;10(4):186-198.

47. Bricca A, Juhl CB, Grodzinsky AJ, Roos EM. Impact of a daily exercise dose on knee joint cartilage – a systematic review and meta-analysis of randomized controlled trials in healthy animals. *Osteoarthr Cartil*. 2017;25(8):1223-1237. doi:10.1016/j.joca.2017.03.009

48. Skoffer B, Dalgas U, Maribo T, Søballe K, Mechlenburg I. No Exacerbation of Knee Joint Pain and Effusion Following Preoperative Progressive Resistance Training in Patients Scheduled for Total Knee Arthroplasty: Secondary Analyses From a Randomized Controlled Trial. *PM&R*. 2018;10(7):687-692. doi:10.1016/j.pmrj.2017.11.002

49. Mononen ME, Tanska P, Isaksson H, Korhonen RK. A Novel Method to Simulate the Progression of Collagen Degeneration of Cartilage in the Knee: Data from the Osteoarthritis Initiative. *Sci Rep*. 2016;6:21415. doi:10.1038/srep21415

50. Sato T, Konomi K, Yamasaki S, Aratani S, Tsuchimochi K, Yokouchi M, et al. Comparative analysis of gene expression profiles in intact and damaged regions of human osteoarthritic cartilage. *Arthritis Rheum*. 2006;54(3):808-817. doi:10.1002/art.21638

51. Yagi R, McBurney D, Laverty D, Weiner S, Horton WE. Intrajoint comparisons of gene expression patterns in human osteoarthritis suggest a change in chondrocyte phenotype. *J Orthop Res*. 2005;23(5):1128-1138. doi:10.1016/j.orthres.2004.12.016

52. Brew CJ, Clegg PD, Boot-Handford RP, Andrew JG, Hardingham T. Gene expression in human chondrocytes in late osteoarthritis is changed in both fibrillated and intact cartilage without evidence of generalised chondrocyte hypertrophy. *Ann Rheum Dis*. 2010;69(01):234-240. doi:10.1136/ard.2008.097139

53. Fukui N, Ikeda Y, Ohnuki T, Tanaka N, Hikita A, Mitomi H, et al. Regional differences in chondrocyte metabolism in osteoarthritis: A detailed analysis by laser capture microdissection. *Arthritis Rheum*. 2008;58(1):154-163. doi:10.1002/art.23175

54. van der Kraan PM, van den Berg WB. Osteophytes: relevance and biology. *Osteoarthr Cartil*. 2007;15(3):237-244. doi:10.1016/j.joca.2006.11.006

55. Uchino M, Izumi T, Tominaga T, Wakita R, Minehara H, Sekiguchi M, et al. Growth Factor Expression in the Osteophytes of the Human Femoral Head in Osteoarthritis. *Clin Orthop Relat Res*. 2000;377(377):119-125. doi:10.1097/00003086-200008000-00017

56. Middleton J, Arnott N, Walsh S, Beresford J. Osteoblasts and osteoclasts in adult human osteophyte tissue express the mRNAs for insulin-like growth factors I and II and the type 1 IGF receptor. *Bone*. 1995;16(3):287-293. doi:10.1016/8756-3282(94)00040-9

SUPPLEMENTAL MATERIAL

Table S1: Primers

Target	Primer name	Sense	Anti-sense
RPLP0	NM_053275.3	GGAAACTCTGCATTCTCGCTTCCT	CCAGGACTCGTTTGTACCCGTTG
GAPDH	NM_002046.4	CCTCCTGCACCACCAACTGCTT	GAGGGGCCATCCACAGTCTTCT
COL2A1	NM_001844.4	ACGTGGAGATCCGGGCAGAG	CGATAACAGTCTTGCCCCACTTACC
ACAN	NM_001135.3	CTCACACAGTCGAAACAGCCACCT	TGTTCCAGCCCTCCTCACATACCTC
COMP	NM_000095.3	TTGATCGCTGTCACAAGCATCTCC	TGACAACTGTCCCCAGAAGAGCAAC
PRG4	NM_005807.6	TCACTGCCCAACATCAGAAAACC	GGATAAGGTCTGCCCAGAACGAG
MMP1	NM_002421.4	CGAATTTGCCGACAGAGATGAAG	GGGAAGCCAAAGGAGCTGTAGATG
MMP3	NM_002422.5	GATCCTGCTTTGTCCTTTGATGCTGT	CTGAGGGATTTGCGCCAAAAGTG
MMP13	NM_002427.4	CCTGATGACGATGTACAAGGGA	TGGCATCAAGGGATAAGGAAGGG
ADAMTS4	NM_005099.6	CCCGCTTCATCACTGACTTCCT	TGCAATGGAGCCTCTGGTTTGT
ADAMTS5	NM_007038.5	ACGCCTTGTGGAAAGGGGAGAA	AGGATCCCCAAGATCCCCAGTT
TNFα	NM_000594.3	TTCCCCAGGGACCTCTCTCTAATC	GAGGGTTTGCTACAACATGGGCTAC
IL1α	NM_000575.5	TGCCCAAGATGAAGACCAACCA	GCCGTGAGTTTCCCAGAAGAAGAG
IL4	NM_000589.4	TCCTGAAACGGCTCGACAGGAAC	ACGTACTCTGGTTGGCTTCCTTCAC
IL6	NM_000600.5	GAGGCACTGGCAGAAAACAACC	CCTCAAACTCCAAAAGACCAGTGATG
IL8	NM_000584.4	CCACACTGCGCCAACACAGAAA	TTCTCCACAACCCTCTGCACCC
IL10	NM_000572.3	CGCTGTCATCGATTTCTTCCCTGT	TGGCTTTGTAGATGCCTTTCTCTTGG
IL13	NM_002188.3	GATTCTGCCCGCACAAGGTCTC	GTAAGAGCAGGTCCTTTACAAACTGGG
IL17A	NM_002190.3	TCCTCAGATTACTACAACCGATCCACC	GCACTTTGCCTCCCAGATCACA
IGF1Ea	NM_000618.3	GACATGCCCAAGACCCAGAAGGA	CGGTGGCATGTCACTCTTCACTC
TGFβ1	NM_000660.5	GAGGTCACCCGCGTGCTAATG	GTCTCTCCCGGCTGCTTGTCC
TGFβ2	NM_003238.4	CCCAAAAGCCAGAGTGCCTGAA	ATGTAGCGCTGGGTTGGAGATG
TGFβ3	NM_003239.4	CTGTGCGTGAGTGGCTGTTGAG	CTCCATTGGGCTGAAAGGTGTG
CTGF	NM_001901.4	TGCGAAGCTGACCTGGAAGAGA	GCCGTCGGTACATACTCCACAGAA
FGF2/bFGF	NM_002006.5	TGACGGGGTCCGGGAGAAGA	ATAGCCAGGTAACGGTTAGCACACAC

Table S2: Number of samples with detected targets

Target	Control group (n=15 participants)			Intervention group (n=16 participants)		
	Central (n=13)†	Submeniscal (n=15)	Osteophyte (n=12)*	Central (n=14)†	Submeniscal (n=16)	Osteophyte (n=10)*
RPLP0	13	15	12	14	16	10
GAPDH	13	15	12	14	16	10
COL2A1	13	15	12	14	16	10
ACAN	13	15	12	14	16	10
COMP	13	15	12	14	16	10
PRG4	13	15	12	14	16	10
MMP-1	13	15	12	14	16	10
MMP-3	1	4	11	ND	2	8
MMP-13	2	7	12	2	10	10
ADAMTS-4	ND	ND	ND	ND	ND	ND
ADAMTS-5 #	6	6	6	9	7	5
TNF-α	ND	ND	6	1	ND	1
IL-1α	ND	ND	ND	ND	ND	ND
IL-4	ND	4	7	ND	1	3
IL-6	ND	ND	ND	ND	ND	ND
IL-8	ND	ND	ND	ND	ND	ND
IL-10	ND	ND	ND	ND	ND	ND
IL-13	ND	ND	ND	ND	ND	ND
IL-17A	ND	ND	ND	ND	ND	ND
IGF1-Ea	8	12	12	8	14	10
TGF-β1	13	15	12	14	16	10
TGF-β2	5	8	11	6	10	8
TGF-β3	10	10‡	12	12	13	10
CTGF	13	15	12	14	16	10
FGF-2/bFGF	13	15	12	14	16	10

Targets in **bold** were sufficiently detected for further analyses. #Less than ten copies detected.
†Two samples resulted in insufficient quality. ‡One sample was lost during analysis. ND: Not detected.
*Not all plateaus had osteophytes to collect. Values are number of samples with detectable target copies.

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PHD-THESIS
DECLARATION OF CO-AUTHORSHIP

The declaration is for PhD students and must be completed for each conjointly authored article. Please note that if a manuscript or published paper has ten or less co-authors, all co-authors must sign the declaration of co-authorship. If it has more than ten co-authors, declarations of co-authorship from the corresponding author(s), the senior author and the principal supervisor (if relevant) are a minimum requirement.

1. Declaration by	
Name of PhD student	Adam El Mongy Jørgensen
E-mail	adam.em.jorgensen@gmail.com
Name of principal supervisor	Michael Kjær
Title of the PhD thesis	Human cartilage growth, regional turnover in vivo, and the effect of exercise in late-stage knee osteoarthritis

2. The declaration applies to the following article	
Title of article	Acute loading has minor influence on human articular cartilage in late-stage knee osteoarthritis: A randomised controlled trial
Article status	
Published ☐ Date:	Accepted for publication ☐ Date:
Manuscript submitted ☐ Date:	Manuscript not submitted ☒
If the article is published or accepted for publication, please state the name of journal, year, volume, page and DOI (if you have the information).	

3. The PhD student's contribution to the article ***(please use the scale A-F as benchmark)*** Benchmark scale of the PhD-student's contribution to the article **A.** Has essentially done all the work (> 90 %) **B.** Has done most of the work (60-90 %) **C.** Has contributed considerably (30-60 %) **D.** Has contributed (10-30 %) **E.** No or little contribution (<10 %) **F.** Not relevant	**A, B, C, D, E, F**
1. Formulation/identification of the scientific problem	B
2. Development of the key methods	F
3. Planning of the experiments and methodology design and development	B
4. Conducting the experimental work/clinical studies/data collection/obtaining access to data	A
5. Conducting the analysis of data	B
6. Interpretation of the results	B
7. Writing of the first draft of the manuscript	A
8. Finalisation of the manuscript and submission	A

Provide a short description of the PhD student's specific contribution to the article.[i]
The PhD student wrote the protocol, obtained ethical permission, and set-up a collaboration with the clinical staff of relevant University Hospital departments of orthopaedic surgery. Further, the PhD student recruited participants, supervised and facilitated the performed intervention, and performed the sampling of biopsies from the cartilage waste tissue obtained from surgery. Finally, the PhD student performed statistics with co-authors, made all the figures, wrote the initial manuscript, and incorporated comments and feedback from co-authors into the final manuscript.

4. Material from another thesis / dissertation[ii]	
Does the article contain work which has also formed part of another thesis, e.g. master's thesis, PhD thesis or doctoral dissertation (the PhD student's or another person's)?	Yes: ☐ No: ☒
If yes, please state name of the author and title of thesis / dissertation.	N/A
If the article is part of another author's academic degree, please describe the PhD student´s and the author´s contributions to the article so that the individual contributions are clearly distinguishable from one another.	N/A

5. Signatures of the co-authors[iii]

	Date	Name	Title	Signature
1.	13/8-20	Peter Schjerling	PhD	
2.	11/8 20	Michael Kjær	DMSc	
3.				
4.				
5.				
6.				
7.				
8.				
9.				
10.				

6. Signature of the principal supervisor

I solemnly declare that the information provided in this declaration is accurate to the best of my knowledge.
Date: 11/8 20
Principal supervisor: Michael Kjær

7. Signature of the PhD student

I solemnly declare that the information provided in this declaration is accurate to the best of my knowledge.
Date:
PhD student: Adam El Mongy Jørgensen 11/8-2020

Please learn more about responsible conduct of research on the Faculty of Health and Medical Sciences' website.

[i] This can be supplemented with an additional letter if needed.
[ii] Please see Ministerial Order on the PhD Programme at the Universities and Certain Higher Artistic Educational Institutions (PhD Order) § 12 (4):
"Any articles included in the thesis may be written in cooperation with others, provided that each of the co-authors submits a written declaration stating the PhD student's or the author's contribution to the work."
[iii] If more signatures are needed please add an extra sheet.

The end

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