Modeling the genetic and mechanical interplay in osteoarthritis: from in vitro systems to mechanistic insights

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CHAPTER 2

A human in vitro 3D neo-cartilage model to explore the response of OA risk genes to hyper-physiological mechanical stress

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Abstract

Objective

Due to the complexity and heterogeneity of osteoarthritis (OA) pathophysiology, studying the interaction between intrinsic molecular changes in chondrocytes after hyper-physiological mechanical stress (MS) and aberrant signalling of OA risk genes remains a challenge. In this study we set out to set up an *in vitro* 3D neo cartilage pellet model that enables us to explore the responses of OA risk genes to hyper-physiological MS.

Design

Human primary chondrocyte neo-cartilage pellets were exposed for 2 days to 2x10 minutes of hyper-physiological dynamic MS attained by a 20% strain and a frequency of 5Hz. In order to assess cartilage damage, sulphated glycosaminoglycan (sGAG) content in the neo-cartilage was quantified using Alcian blue staining and a dimethyl methylene blue (DMMB) assay, while cleavage of aggrecan was visualized by immunohistochemical staining of aggrecan neo-epitope NITEGE. In addition, changes in expression levels of catabolic, anabolic and hypertrophic genes, and of three OA risk genes; *IL11*, *MGP* and *TGFA* were determined.

Results

Hyper-physiological MS induced cartilage damage, as reflected by decreased sGAG content. mRNA levels of aggrecanase *ADAMTS5* were increased, while hypertrophic gene RUNX2 was downregulated. MS increased expression of pro-apoptotic marker *NOXA*. Furthermore, 20% MS led to increased expression of all three OA risk genes *IL11*, *MGP* and *TGFA*.

Conclusions

We established a human *in vitro* model in which hyper-physiological MS induced cartilage damage and catabolic signalling. Next, we demonstrated its usage to study OA risk genes and their response to the mechanical aspects of OA pathophysiology.

Introduction

Although osteoarthritis (OA) is considered a whole joint disease, a major hallmark of OA pathophysiology is progressive degradation of articular cartilage. Articular cartilage, with the chondrocyte being its unique resident cell, is a highly specialized tissue that is laid down during development and assures buffering of mechanical force from articular joints throughout life (*1*). Nonetheless, its vulnerability to OA is highlighted by the poor regenerative capacity of chondrocytes e.g. micro traumas that arise after hyper-physiological mechanical stress. As such, hyper-physiological mechanical stress (MS) on articular cartilage is considered one of the primary factors that triggers onset of OA (*2*).

To identify inherent etiologic factors of OA onset, various comprehensive genome-wide genetic association studies were performed resulting in the identification of multiple strong OA risk genes such as interleukin 11 *(IL11),* transforming growth factor alpha (*TGFA)* and matrix Gla protein (*MGP)*. The functions of these OA genes, suggest that deviations in cartilage maintenance processes are major pathways underlying OA pathology in humans (*3-5*).

To study intrinsic molecular changes in chondrocytes after hyper-physiological load in interaction with aberrant function of OA risk genes, a human *in vitro* model is required that incorporates functional cartilage tissue units and the ability to apply MS, as an important environmental cue to triggering OA-like changes (*6*). Such a model would thus contribute to study intrinsic noxious molecular responses of chondrocytes as well as modifying roles of strong OA risk genes towards efficient risk prediction. Proper understanding of these molecular responses of chondrocytes to MS, could aid in the development of an effective evidence-based framework for OA therapies, and advice on healthy physical activity with and without disease.

To this end, we have exploited a 3D *in vitro* cartilage model of primary human chondrocytes that deposit high quality physiological cartilage (*7*) and induced cartilage damage by hyper-physiological loading. We determined the effects of hyper-physiologic dynamic MS on the extracellular matrix (ECM) and expression of catabolic, anabolic, hypertrophic and apoptotic genes over time. Moreover, we applied the model to gain more insight into how MS affects known OA risk genes, such as *IL11*, *MGP* and *TGFA*.

Methods

Cell culture and mechanical loading of articular neo-cartilage

Human primary chondrocytes were isolated from joints of patients undergoing total joint replacement due to end-stage OA, included in the RAAK study. 3D neo-cartilage pellets were formed using 2.5 x 10^5 chondrocytes and cultured for 11 days in chondrogenic medium. For two consecutive days, the neo-cartilage was subjected to MS (2 x 10 minutes) with 20% compression and a frequency of 5 Hz. For additional details, see online supplementary methods.

Quantification of gene expression

Per donor, RNA from two replicate pellets were pooled and reverse transcribed into cDNA. Gene expression was analysed using RT-qPCR. For additional details, see online supplementary methods.

Histological analysis and immunohistochemistry

Neo-cartilage samples were fixed in 4% formaldehyde and embedded in paraffin. Sections were stained with Alcian Blue and Nuclear Fast Red, or immunohistochemically stained for the aggrecanase-induced neo-epitope NITEGE or IL-11. Relative pixel intensity was determined using ImageJ-Fiji v1.52. For additional details, see online supplementary methods.

Dimethyl Methylene Blue Assay (DMMB) for glycosaminoglycan quantification in neo-cartilage

Sulphated glycosaminoglycan (sGAG) concentrations in the pellets were measured using the Dimethyl Methylene Blue assay. For additional details, see online supplementary methods.

Statistical analysis

All graphs were created using Graphpad Prism 8.0.2 and statistics were performed in IBM SPSS Statistics 25. P-values <0.05 were considered significant. For additional details, see online supplementary methods.

Results

Dynamic hyper-physiological MS in human neo-cartilage increases sGAG release and upregulates ADAMTS5 expression

The mean ± SD age of the donors used in this model was 65 ± 10 years with 11 females and 1 male (**Table S2**). Prior to exposing the neo-cartilage to 20% MS for two consecutive days, we explored the effects of a single 20% MS. As shown in **Fig. S1**, we did not observe any significant changes in Alcian Blue intensity, sGAG content and release after the first 20% MS. Therefore, we subjected the neo-cartilage for two days to 20% MS to increase cartilage damage. As shown in **Fig. 1**, 20% MS significantly

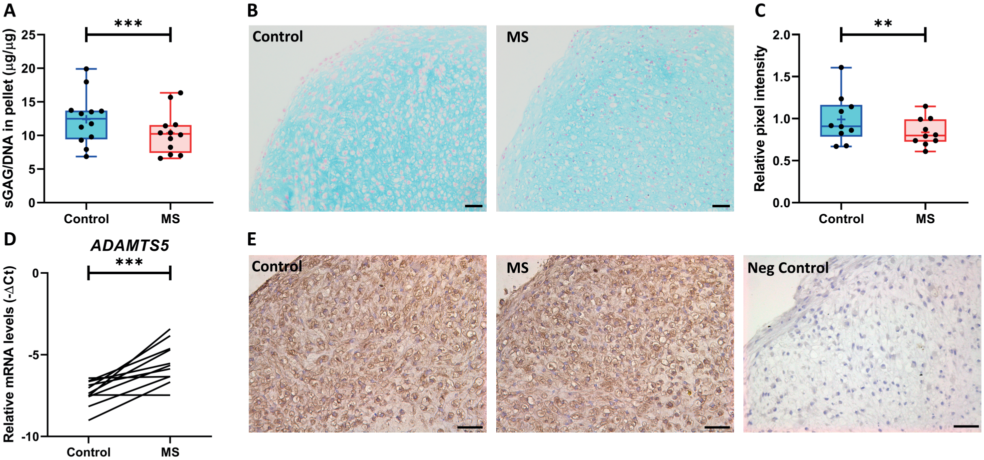

Figure 1 | Assessment of cartilage damage in the neo-cartilage after two days of exposure to 20% MS. (A) sGAG content in the neo-cartilage as determined by DMMB, corrected for DNA content. **(B)** Representative images of Alcian Blue staining and **(C)** subsequent quantification of intensities calculated using Fiji-ImageJ. **(D)** RT-qPCR analysis of catabolic marker *ADAMTS5*. **(E)** Representative images of neo-cartilage stained for aggrecan neo-epitope NITEGE, scale bar = 50 µm. sGAG content and Alcian Blue intensities are presented in a boxplot depicting the median, lower and upper quartiles. Mean is depicted as + and each dot represents a single donor. Lines represent differences in *ADAMTS5* mRNA expression levels between control and 20% MS within a single donor. *P*-values of mean differences in sGAG levels (n=12) and Alcian Blue intensities (n=10) between control and 20% MS were estimated by generalized estimating equations. *P*-values of mean differences in *ADAMTS5* mRNA expression were estimated using a paired samples T-test (n=11). ***$P < 0.001$.

decreased sGAG content (20% MS: 10.37±1.77 µg/µg versus Control: 12.40±2.17 µg/µg, $P < 3.3 \times 10^{-19}$, **Fig. 1A**), which was accompanied by a significant reduction in Alcian Blue intensity (FC=0.82±0.08, $P < 8.0 \times 10^{-3}$, **Figs. 1B and 1C**). However, we did not observe any differences in sGAG concentrations in the medium (**Fig S1B**). On mRNA level, we measured a consistent increase in *ADAMTS5* expression (FC=5.02±3.16, $P=6.0 \times 10^{-4}$, **Fig. 1D and Table S4**). Notably, we observed downregulation of hypertrophic marker *RUNX2* (FC=0.64±0.3, $P=3.6 \times 10^{-2}$, **Table S4**). In contrast, *MMP3*, *MMP13* and *COL10A1* expression did not significantly change (**Table S4**). Furthermore, we observed an upregulation of pro-apoptotic gene *NOXA* (FC=2.46±0.67, $P=8.5 \times 10^{-5}$, **Table S4**). To investigate whether the increased *ADAMTS5* mRNA expression led to aggrecan degradation in the matrix of the neo-cartilage, we performed immunohistochemical staining on the aggrecanase-induced aggrecan neo-epitope NITEGE. As shown in **Fig. 1E**, we showed positive NITEGE staining in the neo-cartilage, localized in the ECM and intracellularly, but were not able to observe differences in expression between control and 20% MS.

20% MS induces expression of OA risk genes

To explore the behaviour of known OA risk genes in neo-cartilage upon 20% MS, we determined expression of three known OA risk genes, *IL11*, *MGP* and *TGFA*. These risk

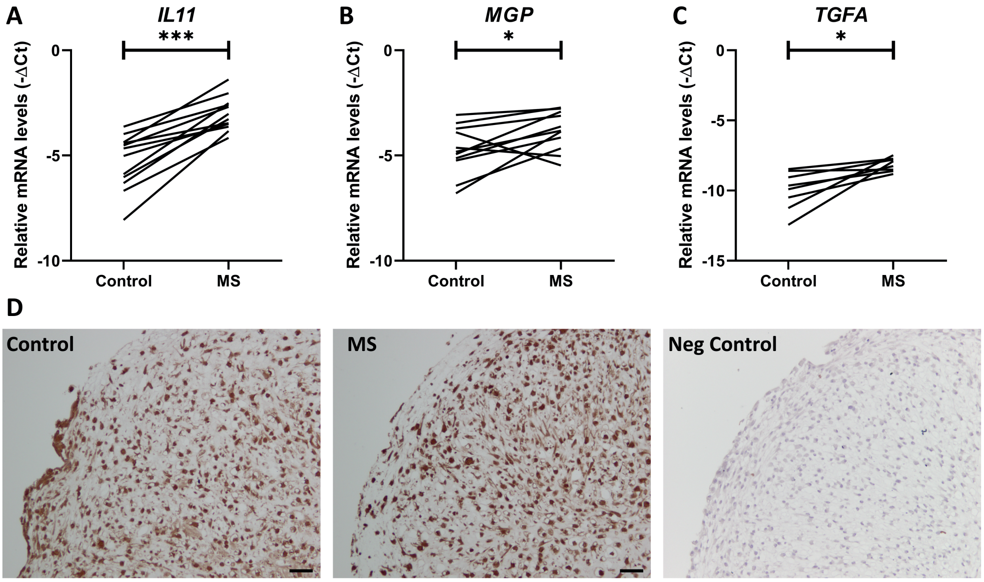

Figure 2 | Gene expression levels of OA risk genes IL11, MGP and TGFA and IL-11 protein expression in the neo-cartilage after two days of exposure to 20% MS. RT-qPCR analysis of (A) *IL11*, **(B)** *MGP* and **(C)** *TGFA*. Lines represent differences in mRNA expression levels between control and MS in individual donors. **(D)** Representative images of neo-cartilage stained for IL-11, scale bar = 50 µm. *P*-values of mean differences in mRNA expression (*n*≥8) were estimated using a paired samples T-test. *P < 0.05, ****P* < 0.001.

genes confer their risk to OA either by subtle increase in gene expression levels during OA, or by alterations in the amino acid sequence of their respective protein, thereby affecting signalling of downstream targets (**Table S1**). As shown in **Fig. 2**, all three genes, *IL11* (FC=6.28±3.12, P=1.2x10^{-5}; **Fig. 2A**), *MGP* (FC=2.52±1.37, P=3.3x10^{-2}; **Fig. 2B**) and *TGFA* (FC=6.27±6.61, P=1.1x10^{-2}; **Fig. 2C**) showed a strong consistent increase in mRNA levels compared to their control. Next, we assessed protein levels of the highest upregulated OA risk gene, *IL11*, in the neo-cartilage. While IL-11 was expressed in the neo-cartilage, no differences were detected between control and 20% MS (**Fig. 2D).**

Discussion

In this study, we present a human *in vitro* neo-cartilage pellet model to study behaviour of OA risk genes after MS-induced cartilage damage. We applied injurious hyper-physiologic dynamic MS to the neo-cartilage that led to catabolic responses by means of upregulation of *ADAMTS5* gene expression, increased apoptosis, and early cartilage damage as a result of proteoglycan loss. Furthermore, the model was then successfully used to confirm that several known OA risk genes showed similar expression patterns in response to MS as what is observed in OA articular cartilage (*8*).

The loading magnitudes of the MS and the microenvironment of the chondrocytes play a key role in the type of response chondrocytes will have (*9*). To initiate a pathophysiological response, we chose dynamical 20% strain (2x10 minutes) at 5 Hz for 2 consecutive days, that was marked by strong significant increase in *ADAMTS5* expression concomitant with loss of sGAGs in the neo-cartilage and increased apoptosis, but without consistent changes in *MMP13* (**Table S4**). This is different to the recently demonstrated effects in long-term 65% mechanically stressed human osteochondral explants that showed a strong increase in *MMP13* expression, but no response of *ADAMTS5* (*10*). This shows that the catabolic responses to mechanical stress strongly depend on the strain, time and type of model being used. Nonetheless, we did not detect any changes in sGAG release. This is possibly due to that during the MS, the neo-cartilage was transferred from the medium to saline and transferred back to the medium afterwards. It is most likely that the observed proteoglycan loss in the neo-cartilage occurred directly during the MS, and was therefore not measurable in the medium. Furthermore, we were not able to measure differences in expression of the aggrecan neo-epitope NITEGE, which might be due to the possibility that the observed *ADAMTS5* upregulation was not yet visible on a protein level.

As cartilage tissue is usually collected during arthroplasty surgery for end stage of OA, the effect of excessive MS on healthy joints (i.e. cartilage) remains unknown. Therefore, researchers turn to human *ex vivo* and *in vitro* models to study the early effects of excessive mechanical loading on chondrocytes and their microenvironment. Over time, various load-based models of OA have been introduced, including monolayer, 3D-and explant cultures, which all differ in chondrocyte state, matrix composition and their response to MS (*11*). In our study, chondrocytes were seeded in high density pellets, they deposit their own ECM that resembles the native cartilage on matrix composition (*7*). Moreover, we have previously demonstrated that this neo-cartilage is highly similar to the autologous cartilage on the epigenetic level, which are crucial mechanisms to dynamically regulate gene expression in articular chondrocytes upon stress and disease (*12*). Furthermore, the primary chondrocytes in this study were collected from macroscopically preserved areas of OA joints. As these cells are in a state between healthy and lesioned OA chondrocytes, they are therefore suitable to study the processes that initiate the transition towards OA. The findings of this study have to be seen in light of some limitations. Since this model is a single cell type model, the interaction between cartilage and underlying bone is lacking. It is known that the subchondral bone plays an important role in the responses on mechanical stress in the articular joint. In contrast to articular cartilage, the neo-cartilage exists of high density seeded chondrocytes, and together with the orientation of the formed collagen fibres, may influence the response on MS. While damage occurred as a result of MS, we did not observe any changes in expression of anabolic genes *COL2A1* and *ACAN*. This might be due to timing or, since the chondrocytes in the neocartilage are continuously producing ECM components, endogenous expression levels are high, which may overrule the effects of the applied MS.

An important part of this study was to explore the responses of three OA risk genes after MS and compare this to what is observed in the articular cartilage of OA patients. *IL11*, *MGP*, and *TGFA* showed significant upregulation upon 20% MS. Similarly, these genes are upregulated in macroscopically lesioned compared to preserved OA cartilage, confirming their role in cartilage maintenance processes, with *IL11* showing the strongest effect in both lesioned OA cartilage and after MS in the current model (*8*). While the downstream effects of IL-11 in MS-induced cartilage damage are still unclear, an important first step is to determine the effects of IL-11 on catabolic and anabolic activity in our model. Styrkarsdottir et al. (*5*) showed an association between a missense mutation in the *IL11* gene, leading to an unstable protein, and higher odds ratios of hip OA. Furthermore, during OA, expression of the IL-11 receptor alpha (*IL11RA)* is downregulated (*8*). Taken together, we postulate that during OA, IL-11 might have protective effects, but may be unable to properly exert its function. *MGP* has previously been identified as a strong OA risk gene (*4*). As a regulator of extracellular calcium levels, low levels of MGP result in higher calcification of the articular cartilage and reduced bone mineral density. While *MGP* is upregulated during OA, the risk allele rs1800801-T is associated with lower expression of *MGP* in articular cartilage (*13*).

In conclusion, we established an *in vitro* model that mimics hyper-physiological dynamic MS-induced cartilage damage in human neo-cartilage, paired with upregulation of *ADAMTS5*. Since the upregulation of *ADAMTS5* was specific, we propose this model as a suitable platform for high throughput screening of potential drugs targeted against *ADAMTS5*. Furthermore, we demonstrated that this model can be used to study the behaviour of OA risk genes and how they may relate to the mechanical aspects of OA pathophysiology.

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Contributions

Study concept and design: Ritchie G.M. Timmermans, Niek G.C. Bloks, Peter M. van der Kraan, Martijn H. J. van den Bosch, Arjen B. Blom, Yolande F.M. Ramos and Ingrid Meulenbelt
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Data analysis: Ritchie G.M. Timmermans, Niek G.C. Bloks, Yolande. F.M. Ramos and Ingrid Meulenbelt
Preparation of the manuscript: Ritchie G.M. Timmermans, Niek G.C. Bloks, Yolande. F.M. Ramos and Ingrid Meulenbelt
Critical reviewing and approval of the manuscript: All authors

Role of the funding source

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Competing interests

All authors declare that they have nothing to disclose.

Compliance with Ethics Guidelines

The RAAK study has been approved by the medical ethical committee of the Leiden University Medical Center (P08.239/P19.013). Written informed consent was obtained from all patients, and patients had the right to withdraw at any time.

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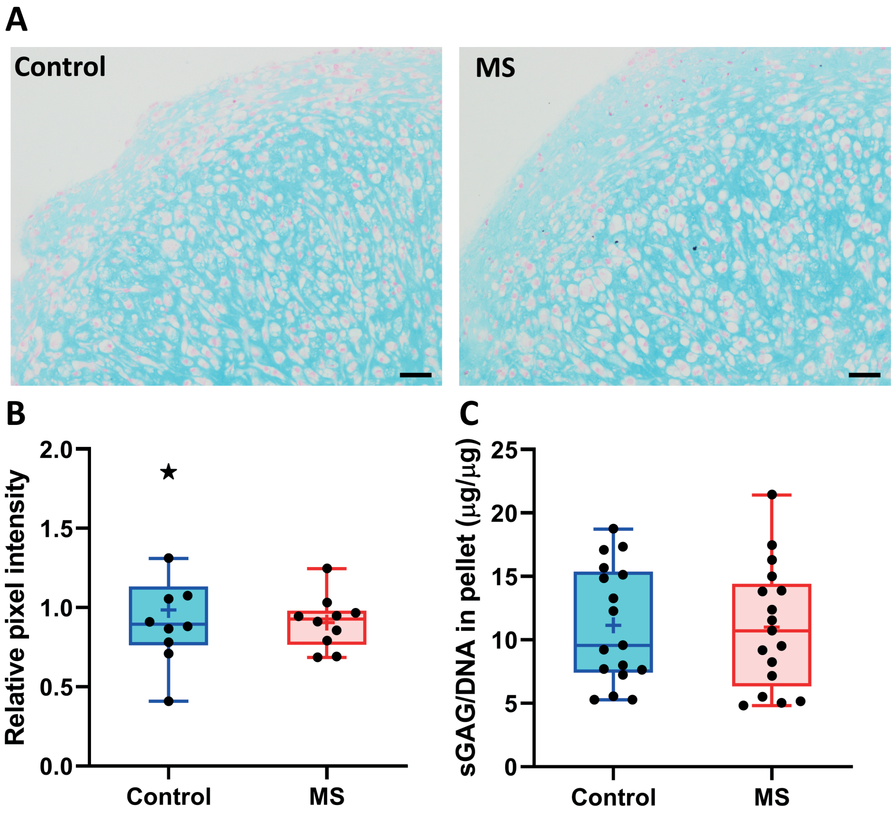
Supplementary Materials



Supplementary Figure S1 | Assessment of damage in the neo-cartilage after the first day of 20% MS. (A-B) Representative images of Alcian Blue staining and quantification of Alcian Blue intensity calculated using Fiji-ImageJ. Scale bar = 50 µm. **(C)** sGAG content in the neo-cartilage as determined by DMMB. Data are presented in a boxplot depicting the median, lower and upper quartiles. Mean is depicted as +, each dot represents a single donor, and * represents a statistical outlier. P-values of mean differences in Alcian Blue intensities (N = 10) and sGAG levels (N = 17) between controls and MS neo-cartilage were estimated by generalized estimating equations (GEE).

Supplementary Table S1 **– SNPs conferring risk to OA, and their associated genes, location, amino acid change, phenotype, effector allele (EA), minor allele frequency (MAF), odds ratio and effect.**

SNP	Gene	Location	Amino-acid change	Phenotype	EA	MAF	Odds Ratio	Expression in OA articular cartilage	Effect	Refs
rs4252548	*IL11*	Exonic	p.Arg112His	Hip OA	T	0.01	1.3 (1.2-1.4)	FC = 22.8, P = 1,5x10^{-20}	Reduced thermal stability	[1-3]
rs4764133	*MGP*	Intergenic	-	Hand OA	T	0.28	1.09 (1.04-1.13)	FC = 1.44, P = 2.1x10^{-2}	In high LD with rs1800801 (R^2 = 0.95) and rs4236 (R^2 = 0.86)	[1, 4, 5]
				Finger OA			1.12 (1.07-1.17)			
				Thumb OA			1.07 (1.03-1.12)			
rs1800801	*MGP*	Intergenic	-	-	T	0.26	-	FC = 1.44, P = 2.1x10^{-2}	Allelic imbalance in OA	[1, 5]
rs4236	*MGP*	Exonic	p.Thr127Ala	Hip OA	C	0.39	-	FC = 1.44, P = 2.1x10^{-2}	Allelic imbalance in OA	[1, 5]
rs2862851	*TGFA*	Intronic	-	Hip and knee OA	T	0.39	1.41	FC = 1.50, P = 1.3x10^{-3}	-	[1, 6, 7]

Supplementary Table S2 – Patient characteristics

Characteristic	One day 20% MS (N=17)	Two days 20% MS (N=12)
Sex-no (%)		
Female	15 (88%)	11 (92%)
Male	2 (12%)	1 (8%)
Age-yr		
Mean (SD)	67 (9)	65 (10)
Range	52-86	52-86

Supplementary Table S3 – Primer sequences

Gene (primer)	Forward sequence	Reverse sequence
GAPDH	5'-TGCCATGTAGACCCCTTGAAG-3'	5'-ATGGTACATGACAAGGTGCGG-3'
SDHA	5'-TGGAGCTGCAGAACCTGATG-3'	5'-TGTAGTCTTCCCTGGCATGC-3'
MMP3	5'-GAGGCATCCACACCCTAGGTT-3'	5'-TCAGAAATGGCTGCATCGATT-3'
MMP13	5'-TTGAGCTGGACTCATTGTCG-3'	5'-GGAGCCTCTCAGTCATGGAG-3'
ADAMTS5	5'-TGGCTCACGAAATCGGACAT-3'	5'-GCGCTTATCTTCTGTGGAACC-3'
COL2A1	5'-CTACCCCAATCCAGCAAACGT-3'	5'-AGGTGATGTTCTGGGAGCCTT-3'
ACAN	5'-AGAGACTCACACAGTCGAAACAGC-3'	5'-CTATGTTACAGTGCTCGCCAGTG-3'
SOX9	5'-CCCCAACAGATCGCCTACAG-3'	5'-CTGGAGTTCTGGTGGTCGGT-3'
RUNX2	5'-TGTGGTTACTGTCATGGCG-3'	5'-AGGTAGCTACTTGGGGAGGA-3'
COL10A1	5'-GGCAACAGCATTATGACCCA-3'	5'-TGAGATCGATGATGGCACTCC-3'
IL11	5'-CTCTACAGCTCCCAGGTGTGC-3'	5'-AGGTAGGACAGTAGGTCCGCT-3'
MGP	5'-CGCCCCCAGATTGATAAGTA-3'	5'-TCTCCTTTGACCCTCACTGC-3'
TGFA	5'-GCAGGTTTTTGGTGCAGGAG-3'	5'-GGTGATGGCCTGCTTCTTCT-3'
NOXA	5'-GGAGATGCCTGGGAAGAAG-3'	5'-CCTGAGTTGAGTAGCACACTCG-3'
BAK1	5’-GGGGACGACATCAACCGACGC-3’	5’-CGAAGCGGGTCACCTGGCCTA-3’
CASP7	5’-GCTGACTTCCTCTTCGCCTA-3’	5’-CAAACCAGGAGCCTCTTCCT-3’
*BIRC5 (*Survivin*)*	5'-AGCCCTTTCTCAAGGACCA-3'	5'-CAGCTCCTTGAAGCAGAAGAA-3'

Supplementary Table S4 – Gene expression in neo-cartilage pellets after one and two days of 20% MS. RT-qPCR analysis of catabolic markers MMP3, MMP13 and ADAMTS5, anabolic markers COL2A1, ACAN and SOX9, hypertrophic markers RUNX2 and COL10A1, and apoptotic genes NOXA and SURVIVIN, comparing one day of 20% MS (n=17 donors) and two days of 20% MS (n=11 donors) to their respective controls. Fold changes were calculated using the 2$^{-\Delta\Delta Ct}$ method. Statistical differences between –ΔCt values of control and MS groups were determined using a paired sample T-test.

	One day 20% MS								Two days 20% MS							
Gene	Fold Change	FC 95% CI		Mean	Std. Error Mean	95% CI		*P*-value	Fold Change	FC 95% CI		Mean	Std. Error Mean	95% CI		*P*-value
		Lower	Upper			Lower	Upper			Lower	Upper			Lower	Upper	
Catabolic																
MMP3	2.05	1.23	2.88	0.70	0.24	0.18	1.22	**1.1x10^{-2}**	1.23	0.71	1.75	0.08	0.22	-0.41	0.57	7.2x10^{-1}
MMP13	1.15	0.72	1.58	-0.21	0.31	-0.87	0.45	5.1x10^{-1}	0.78	0.50	1.07	-0.61	0.28	-1.23	0.01	5.4x10^{-2}
ADAMTS5	4.18	2.60	5.75	1.77	0.26	1.20	2.33	**1.4x10^{-5}**	5.02	1.86	8.18	1.84	0.38	1.00	2.68	**6.0x10^{-4}**
Anabolic																
COL2A1	0.95	0.58	1.32	-0.57	0.35	-1.31	0.17	1.2x10^{-1}	0.95	0.49	1.41	-0.43	0.36	-1.23	0.38	2.6x10^{-1}
ACAN	1.14	0.51	1.76	-0.67	0.42	-1.57	0.23	1.3x10^{-1}	1.56	0.26	2.86	-0.56	0.65	-2.01	0.89	4.1x10^{-1}
SOX9	1.15	0.34	1.96	-0.47	0.46	-1.50	0.56	3.4x10^{-1}	2.66	0.60	4.72	0.80	0.53	-0.46	2.07	1.8x10^{-1}
Hypertrophic																
RUNX2	1.05	0.41	1.69	-0.36	0.35	-1.15	0.43	3.3x10^{-1}	0.64	0.34	0.94	-0.75	0.26	-1.43	-0.07	**3.6x10^{-2}**
COL10A1	1.12	0.41	1.83	-0.51	0.40	-1.38	0.36	2.2x10^{-1}	1.38	0.70	2.06	0.30	0.28	-0.39	0.99	3.3x10^{-1}
***Pro*-apoptotic**																
NOXA	-	-	-	-	-	-	-	-	2.50	1.90	3.10	1.21	0.19	0.78	1.64	**8.5x10^{-5}**
BAK1	-	-	-	-	-	-	-	-	1.63	0.30	2.96	0.90	0.62	-0.49	2.29	1.8x10^{-1}
CASP7	-	-	-	-	-	-	-	-	1.59	1.01	2.17	0.45	0.26	-0.14	1.04	1.2x10^{-1}
Anti-apoptotic																
BIRC5 (Survivin)	-	-	-	-	-	-	-	-	1.76	0.19	3.33	-0.12	0.59	-1,43	1.18	8.3x10^{-1}

***Supplementary Table S5* – Gene expression in neo-cartilage pellets after the two days of 20% MS**. RT-qPCR analysis of *IL11*, *MGP* and *TGFA* comparing 20% MS to their respective controls (*n*≥8 donors). Fold changes were calculated using the $2^{-\Delta\Delta Ct}$ method. Statistical differences between –ΔCt values of control and MS groups were determined using a paired sample T-test.

	20% MS							
Gene	**Fold Change**	**FC 95% CI**		**Mean**	**Std. Error Mean**	**95% CI**		**P-value**
		Lower	**Upper**			**Lower**	**Upper**	
IL11	6.28	3.16	9.40	2.29	0.30	1.62	2.96	**1.2×10^{-5}**
MGP	2.52	1.15	3.89	0.91	0.37	0.09	1.73	**3.3×10^{-2}**
TGFA	6.27	-0.34	12.88	1.84	0.54	0.57	3.12	**1.1×10^{-2}**

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Supplemental Methods

Cell culture and mechanical loading of articular neo-cartilage

The effects of hyper-physiological MS were investigated using our previously described human 3D *in vitro* neo-cartilage (*1*). In short, chondrocytes were isolated from preserved areas of joints of patients undergoing total joint replacement due to end-stage OA included in the Research in Articular Osteoarthritis Cartilage (RAAK) study (*2*). The age range of the patients was 52-68 years, with an average (SD) age of 65 (10) years, and mostly female (92%, Table S2). Cells were expanded for two passages and pellets (250,000 cells/pellet) were created by centrifugation. The following day, pellets were incubated in chondrogenic medium to induce chondrogenesis. Within 11 days, the chondrocytes have deposited high quality neo-cartilage and were subjected to MS using a Mach-1 mechanical testing system (Biomomentum, Laval, Canada). MS was applied twice for 10 minutes, with a frequency of 5 Hz, a strain of 20% and a recovery interval of 10 minutes at two consecutive days. Samples were collected 12 hours after MS.

RT-qPCR

Per donor, two replicate neo-cartilage pellets were collected in TRIzol (Invitrogen™, Carlsbad, CA, USA) and RNA was isolated using the RNeasy Mini Kit (Qiagen, Venlo, the Netherlands) according to manufacturer’s protocol. DNA contamination was removed by treating the RNA with RNase-Free DNase (Qiagen, Venlo, the Netherlands). RNA quality (A260/280: 1.7-2.0) was assessed using the Nanodrop. RNA concentrations were measured with the Qubit® 2.0 Fluorometer (Invitrogen™, Carlsbad, CA, USA) using the RNA HS Assay Kit (Invitrogen™, Carlsbad, CA, USA)., respectively, with an A260/280 between 1.7-2.0. RNA was reverse transcribed into cDNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche, Basel, Switzerland). cDNA was amplified using FastStart SYBR Green Master (Roche, Basel, Switzerland) and mRNA expression was measured in triplicates in a MicroAmp™ Optical 384-Well Reaction Plate (ThermoFisher Scientific, Landsmeer, the Netherlands), using the QuantStudio™ Flex Real-Time PCR system (Applied Biosystems™, Foster City, CA, USA), with the following cycling conditions: 10 min 95 °C; 10 sec 95 °C, 30 sec 60 °C, 20 sec 72 °C (45 cycles); 1 min 65 °C and 15 sec 95 °C . Primer efficiency was tested using a cDNA dilution series, and primers were considered efficient with an efficiency between 90% and 110%. -ΔCt expression levels were calculated using two housekeeping genes *GAPDH* and *SDHA*, with the following formula: *ΔCt = Ct (gene of interest) – Ct (average housekeeping genes)*. Both housekeeping genes were stably expressed in this model. Fold changes were calculated using the $2^{-\Delta\Delta Ct}$ method with *ΔΔCt = ΔCt (MS) – ΔCt (Control)*. Primer sequences are listed in Table S3.

Histological analysis and immunohistochemistry

Neo-cartilage samples were fixed in 4% formaldehyde and embedded in paraffin. Sections were stained with Alcian Blue (Sigma-Aldrich, Zwijndrecht, the Netherlands) and Nuclear Fast Red (Sigma-Aldrich, Zwijndrecht, the Netherlands). Microscopic images were loaded into ImageJ-Fiji v1.52. Images were split into three colour channels, selecting the channel containing the Alcian Blue staining. Images were corrected for uneven illumination using the rolling ball algorithm. The average pixel intensity was measured, corrected for background, and relative pixel intensities were calculated using the control as reference group. Aggrecanase activity and localization of IL-11 in the neo-cartilage was visualized immunohistochemically. Sections were stained for the aggrecanase-induced neo-epitope NITEGE as described previously (*3*). In short, antigen retrieval was done by treating deparaffinized sections with hyaluronidase (10 mg/ml, Sigma, Zwijndrecht, the Netherlands) and a citrate buffer (10 mM, pH 6.0). Sections were incubated overnight with a primary antibody raised against the NITEGE neo-epitope (5 µg/ml), a kind gift from John Mort (McGill University, Montreal, Canada), or a rabbit IgG control (5 µg/ml, Agilent, Santa Clara, CA, USA), followed by incubation with VECTASTAIN® Elite ABC-HRP Kit (Vector Laboratories, Burlingame, CA, USA). For IL-11, antigen retrieval was done by treating deparaffinized sections with proteinase K (5 µg/ml, Qiagen, Venlo, The Netherlands) and hyaluronidase (5 mg/ml, Sigma, Zwijndrecht, the Netherlands). Sections were incubated overnight with a primary antibody raised against human IL-11 (1:100, ThermoFisher Scientific, Landsmeer, the Netherlands), followed by incubation with a HRP conjugated secondary antibody (ImmunoLogic, Duiven, the Netherlands). Peroxidase binding for both IL-11 and NITEGE was visualized using diaminobenzidine, and sections were counterstained with haematoxylin.

Dimethyl Methylene Blue Assay (DMMB) for glycosaminoglycan quantification in neo-cartilage

Sulphated glycosaminoglycan (sGAG) concentrations in the neo-cartilage (µg sGAG/µg DNA) and in the medium (µg SGAG/ml medium) was measured using the Farndale Dimethyl Methylene Blue (DMMB, Sigma, Zwijndrecht, the Netherlands) method (*4*). Chondroitin sulphate (Sigma, Zwijndrecht, the Netherlands) was used as a reference standard. Absorbance was measured at 535 and 595 using a microplate reader (Synergy HT, Biotek, Winooski, VT, USA). Neo-cartilage sGAG concentrations were corrected for DNA content measured with the Qubit® 2.0 Fluorometer (Invitrogen™, Carlsbad, CA, USA) using the dsDNA HS Assay Kit (Invitrogen™, Carlsbad, CA, USA).

Statistical analysis

All graphs were created using GraphPad Prism 8.0.2. -ΔCt line graphs were created to show the direction of effect of mRNA levels from individual donors. All data was tested for normal distribution using the Shapiro-Wilk test. Statistical differences in -ΔCt expression levels were analysed using a paired *t*-test. Statistical significance of mean differences in Alcian Blue intensities, neo-cartilage and medium sGAG levels were estimated using a generalized estimating equation (GEE). Since perfect pairs were absent, by applying a GEE, we could adjust for dependencies among donors by adding a random effect for sample donors. We followed a linear GEE model, with Alcian Blue intensities and sGAG levels as dependent variable and condition as a factor. Statistics were performed in IBM SPSS Statistics 25. *P*-values < 0.05 were considered significant.

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