Aged human osteochondral explants as biomimetic osteoarthritis model: towards a druggable target in osteoarthritis

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Chapter 4

Characterization of dynamic changes in Matrix Gla Protein (*MGP*) gene expression as function of genetic risk alleles, osteoarthritis relevant stimuli, and the vitamin K inhibitor warfarin

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Abstract

Objective

We here aimed to characterize changes of Matrix Gla Protein (*MGP*) expression in relation to its recently identified OA risk allele rs1800801-T in OA cartilage, subchondral bone and human *ex vivo* osteochondral explants subjected to OA related stimuli. Given that MGP function depends on vitamin K bioavailability, we studied the effect of frequently prescribed vitamin K antagonist warfarin.

Methods

Differential (allelic) mRNA expression of *MGP* was analyzed using RNA-sequencing data of human OA cartilage and subchondral bone. Human osteochondral explants were used to study exposures to interleukin 1 beta (IL-1β; inflammation), triiodothyronine (T3; Hypertrophy), warfarin, or 65% mechanical stress (65%MS) as function of rs1800801 genotypes.

Results

We confirmed that the *MGP* risk allele rs1800801-T was associated with lower expression and that *MGP* was significantly upregulated in lesioned as compared to preserved OA tissues, mainly in risk allele carriers, in both cartilage and subchondral bone. Moreover, *MGP* expression was downregulated in response to OA like triggers in cartilage and subchondral bone and this effect might be reduced in carriers of the rs1800801-T risk allele. Finally, warfarin treatment in cartilage increased *COL10A1* and reduced *SOX9* and *MMP3* expression and in subchondral bone reduced *COL1A1* and *POSTN* expression.

Discussion & conclusions

Our data highlights that the genetic risk allele lowers *MGP* expression and upon OA relevant triggers may hamper adequate dynamic changes in *MGP* expression, mainly in cartilage. The determined direct negative effect of warfarin on human explant cultures functionally underscores the previously found association between vitamin K deficiency and OA.

Keywords

Osteoarthritis**,** Articular cartilage, Subchondral bone, Matrix Gla Protein, Warfarin, Vitamin K, Genetic risk

Introduction

Osteoarthritis (OA) is the most common degenerative disease of joints and its incidence is rising with increasing obesity and age, resulting in a high social and economic burden on society. Interacting risk factors for OA include obesity, age, sex, abnormal loading and genetic factors. The genetic component of OA is estimated to be in the range of 40%-60% [1,2]. For that matter, large-scale genome wide association studies (GWAS) have identified strong, in other words highly significant and reproducible, OA risk genes involved in the aetiology of OA, whereas follow-up studies have shown that risk single nucleotide polymorphisms (SNPs) frequently modulate pathology due to altering transcription of the genes in *cis* both in bone and cartilage [3-6].

In this regard Matrix Gla protein (*MGP*) via rs4764133 [7] with proxy SNPs rs1800801 and rs4236 [8], was previously identified as strong OA risk gene for hand OA with the OA conferring allele associated with lower expression of *MGP* relative to the non-risk allele [7] in a range of joint tissues but its effect was most profound in cartilage and subchondral bone [7,9]. On the other hand, these studies could not identify significant differential expression of *MGP* in OA pathophysiology in macroscopically lesioned OA compared to preserved cartilage [7] nor in macroscopically preserved cartilage compared to healthy cartilage [9]. These differential expression analyses were, however, determined in a relatively small sample size.

MGP regulates extracellular calcium levels via high affinity to its γ-carboxyglutamic acid (Gla) residues. Low *MGP* levels results in higher calcification of cartilage tissue and a reduced bone mineral density [10-12]. As the OA risk allele (rs1800801) has been associated with a reduced *MGP* gene expression [12] and with increased vascular calcification [13], this would suggest increased cartilage calcification in carriers of the OA risk allele. The latter was further justified by recapitulating downregulation of *MGP* in cartilage chondrocytes resulting in pro-catabolic (*ADAMTS4, MMP13*), as well as pro-hypertrophic *(COL10A1, VEGFA*) mRNA signalling [9]. The MGP protein is produced by the cell in inactive form and is dependent on vitamin K for activation, via carboxylation (c-MGP). As such, low vitamin K levels have been hypothesized to play a role in OA pathogenesis [14,15]. Similarly, vitamin K antagonists such as warfarin, that are frequently prescribed for the prevention of thromboembolic events in patients with atrial fibrillation [16], have been suggested to predispose to OA [17]. Nonetheless, the direct effect of warfarin on human articular cartilage tissue homeostasis has not been assessed.

Here we set out to explore *MGP* gene expression in relation to the OA risk allele rs1800801-T, in a large RNA-sequencing dataset containing both macroscopically preserved and lesioned cartilage [18] and subchondral bone [19] as well as in our recently established full thickness human *ex vivo* osteochondral explant model [20]. The latter allowing us to study the effect of the OA risk allele on the dynamic *MGP* response to different OA related stimuli, such as inflammation (Interleukin 1 beta (IL-1β)), hypertrophy (Triiodothyronine (T3)) and 65% mechanical stress (65%MS). Moreover, we used the human *ex vivo* explant model to study the direct effect of vitamin K antagonist, warfarin, on articular cartilage and subchondral bone homeostasis.

Material and Methods

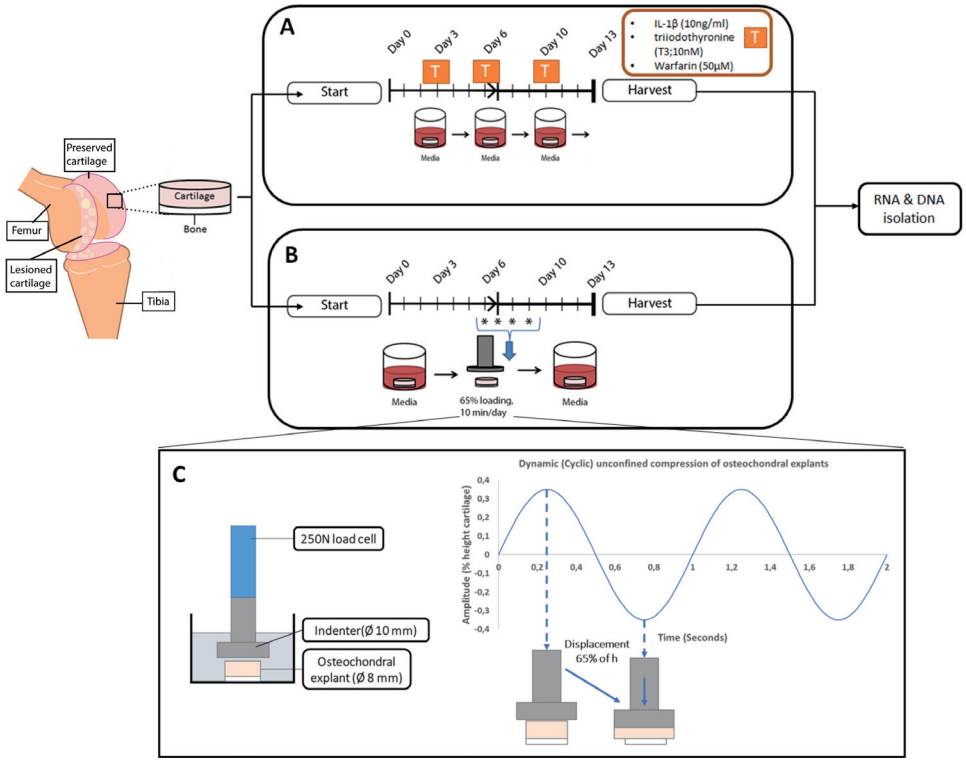

Figure 1 | Schematic representation of different perturbations applied to osteochondral explants. Osteochondral explants were punched from the still macroscopically preserved looking knee condyle area and taken into culture. **[A]** Explants were subjected to treatment with IL-1β (10 ng/ml), triiodothyronine (T3; 10 nM) or warfarin (50 μM). **[B]** Explants received mechanical stresses at a strain of 65% for 10 minutes per day on four subsequent days. On day 13, cartilage and bone was separated, snap frozen and stored at -80C. **[C]** Schematic representation of the dynamic (cyclic) compression applied to osteochondral explants. Legend: N=Newton; h=height.

Sample description

Human material was obtained from the Research in Articular Osteoarthritis Cartilage (RAAK) biobank as previously described in detail [21]. The RAAK study is approved by the medical ethics committee of the Leiden University Medical Center (P08.239/P19.013). In this study, RNA-sequencing data was included of paired macroscopically preserved and lesioned OA cartilage of N=35 participants [18] and subchondral bone of N=24 participants [19] for which sample characteristics have previously been described. In total 136 osteochondral explants were harvested from the macroscopically preserved condyle knee joints of N=18 participants and divided over the different experiment (**Supplementary Figure 1**). Multiple osteochondral explants containing both cartilage and bone (diameter of 8 mm) were extracted per participant and washed in sterile PBS before taking into culture. Donor characteristics of osteochondral explants are described in **Supplementary Table 1** and study design is described in **Supplementary Figure 1**. For additional details on neo-cartilage deposition, RNA and

DNA isolations, TaqMan genotyping, RNA sequencing data of cartilage and subchondral bone, unconfined dynamic (cyclic) compression, expression quantitative trait loci (eQTL), Allelic Expression Imbalance (AEI) and data analysis, see the **Supplementary Methods**.

Treatment of osteochondral explants

Explants were cultured in 24 wells plates (Greiner CELLSTAR; Sigma) supplemented with 1.5 ml CDM in a 5% (v/v) CO_2 incubator at 37°C. Three days after extraction, explants were treated with either IL-1β (10 ng/ml), triiodothyronine (T3, 10 nM; Sigma) or warfarin (50 μM; Sigma), depicted in **Figure 1A**. Six days after extraction, dynamic unconfined compression was applied to explant tissues using the Mach-1 mechanical testing system (Biomomentum Inc., Laval, QC, Canada) on four subsequent days **(Figure 1B)**. Mechanical stress was applied at a strain of 65% of cartilage height and at a frequency of 1 Hz, mimicking walking speed (**Figure 1C**). Cartilage and bone were separated using a scalpel, snap-frozen in liquid nitrogen, and stored at -80°C for RNA isolation.

Reverse transcription and Real-Time PCR

Real-Time PCR for gene expression was performed with QuantStudio 6 Real-Time PCR system (Applied Biosystems) using Fast Start Sybr Green Master mix (Roche Applied Science). Primer sequences (**Table 1**) used were tested for linear amplification and missing datapoints for genes are summarized in **Supplementary Table 2** and **3**. Details on normalization can be found in the **Supplementary Methods**.

Table 1. Primer sequence used to determine gene expression levels in real-time PCR.

Gene name	Forward 5'-3'	Reverse 5'-3'
SDHA	TGGAGCTGCAGAACCTGATG	TGTAGTCTTCCCTGGCATGC
MGP	CGCCCCCAGATTGATAAGTA	TCTCCTTTGACCCTCACTGC
SOX9	CCCCAACAGATCGCCTACAG	CTGGAGTTCTGGTGGTCGGT
ACAN	AGAGACTCACACAGTCGAAACAGC	CTATGTTACAGTGCTCGCCAGTG
COL2A1	CTACCCCAATCCAGCAAACGT	AGGTGATGTTCTGGGAGCCTT
RUNX2	CTGTGGTTACTGTCATGGCG	AGGTAGCTACTTGGGGAGGA
ALPL	CAAAGGCTTCTTCTTGCTGGTG	CCTGCTTGGCTTTTCCTTCA
COL1A1	GTGCTAAAGGTGCCAATGGT	ACCAGGTTCACCGCTGTTAC
COL10A1	GGCAACAGCATTATGACCCA	TGAGATCGATGATGGCACTCC
MMP3	GAGGCATCCACACCCTAGGTT	TCAGAAATGGCTGCATCGATT
MMP13	TTGAGCTGGACTCATTGTCG	GGAGCCTCTCAGTCATGGAG
ADAMTS5	TGGCTCACGAAATCGGACAT	GCGCTTATCTTCTGTGGAACC
COMP	ACAATGACGGAGTCCCTGAC	TCTGCATCAAAGTCGTCCTG
OMD	GGACACAACAAATTGAAGCAAGC	TGGTGGTAATGTAGTGGGTCA
BGLAP	CCCTCCTGCYYGGACACAAA	CACACTCCTCGCCCTATTGG
OGN	TGATGAAATGCCCACGTGTC	TTTGGTAAGGGTGGTACAGCA
SPP1	GCCAGTTGCAGCCTTCTCA	AAAAGCAAATCACTGCAATTCTCA
TNFRSF11B	TTGATGGAAAGCTTACCGGGA	TCTGGTCACTGGGTTTGCATG
BMP2	TCCATGTGGACGCTCTTTCA	AGCAGCAACGCTAGAAGACA
POSTN	TACACTTTGCTGGCACCTGT	TTTAAGGAGGCGCTGATCCA

Statistical analyses

Differential *MGP* expression analyses between preserved and lesioned OA cartilage and bone including false discovery rates (FDR) as multiple testing correction for the genome wide analyses were reproduced from Coutinho [18] and Tuerlings [19], respectively. Description on their study design and sample numbers are in **Supplementary Methods**. To assess allelic expression imbalance (AEI) we applied our previously published methodology in R [22] to RNA sequence data of *MGP* in the larger dataset of cartilage [18] and a dataset of bone [19] which is further outlined in **Supplementary Methods**. To test expression quantitative trait analyses (eQTL) and differential expression of *MGP* in genotype strata in the current manuscript, we used the variance stabilizing transformation (VST) normalized *MGP* expression levels of these RNA sequencing datasets and used generalized estimating equations (GEE) [23] to effectively adjust for dependencies of genotypes among donors by adding a random effect for sample donor. Details of the models applied are outlined in **Supplementary Methods**. *MGP* expression by RT-qPCR in the *in vitro* 3D-neo cartilage formation was estimated using a generalized linear mixed model (GLMM) using *MGP* levels (-ΔCT) as dependent variable and time as repeated measure: *MGP level ~ Time + (1|Donor)*. In the osteochondral explant models fold changes (FC) of RT-qPCR expression were determined by calculating the log 2 of the -ΔΔCt for each sample ($2^{-\Delta\Delta CT}$) where FC>1 is upregulation and FC<1 is downregulation of treated samples compared to control samples. The reported P-values were determined by applying GEE to -ΔCT values to effectively adjust for dependencies among donors of the explants by adding a random effect for sample donor as we did not have perfect pairs for each analysis. We followed a linear GEE model, with MGP level as dependent variable, treatment as factor and exchangeable working matrix: *MGP level ~ Treatment + (1|Donor)* [24]. Differences in effect sizes between strata was determined by performing unpaired student's t-test on the fold changes corrected for control samples. Warfarin treated osteochondral explants samples were paired hence a paired sample t-test was performed to determine between-group differences and p-values. Except for AEI, Statistical analyses were performed in SPSS statistics 23 (IBM). Outliers were investigated using Grubbs's test and normal distribution was determined using Shapiro-Wilk test and visually inspecting Q-Q plots. The boxplots represent 25th, 50th and 75th percentiles, and whiskers extend to the 95%CI.

Results

Expression patterns of MGP in previous established RNA sequencing datasets of preserved and lesioned OA cartilage and subchondral bone

We used our previously established RNA sequencing dataset of macroscopically preserved and lesioned OA cartilage samples (N=35 pairs [18]) and subchondral bone (N=24 pairs [19]), to examine differential *MGP* expression with OA tissue status and with the OA risk SNPs (see **Supplementary Methods and Figures**). An increased expression of *MGP* in lesioned compared to preserved OA cartilage was observed (FC=1.45, 95%CI[1.24;1.61], P-value=1.78×10^{-3}) and this increase of *MGP* was genome wide significant (FDR=0.021). Similarly, *MGP* was upregulated in lesioned compared to preserved OA subchondral bone (FC=1.53, 95%CI[1.22;1.64] , P-value=0.023), but this was not genome wide significant (FDR=0.12). Together, these results show a robust upregulation of *MGP* expression with ongoing OA pathophysiology.

Here we studied whether the *MGP* differential expression between preserved and lesioned OA tissues was affected by *MGP* OA risk allele carriership. As shown in **Figure 2A,** the *MGP* upregulation occurs particularly among risk allele carriers rs1800801-T in lesioned compared to preserved OA cartilage independent of age and sex of donors (OR=2.70, 95%CI[1.16;6.29], P-value=0.021). Notably however, the overall *MGP* expression remains lower among risk allele carriers rs1800801-T as compared to carriers of the reference allele rs1800801-C. The same effect was observed in subchondral bone, where *MGP* was found to be upregulated in lesioned compared to preserved tissue only in risk allele carriers rs1800801-T (OR=3.04, 95%CI[1.24;7.45], P-value=0.015) independent of age and sex of donors (**Figure 2B**).

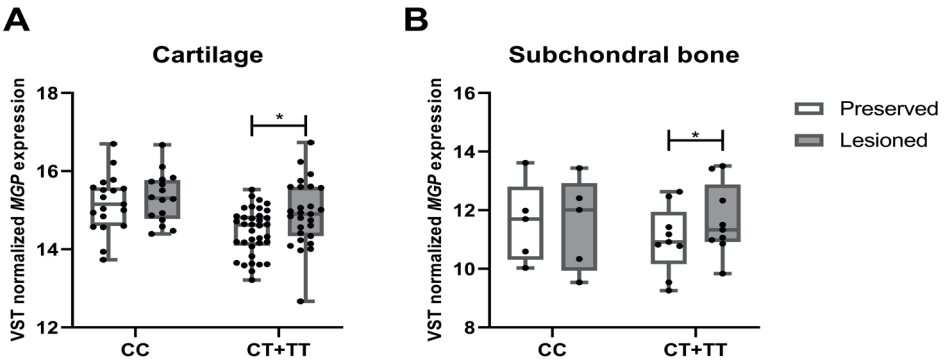

Figure 2 | MGP expression as function of the transcript and OA risk SNP rs1800801. [A] Variance stabilizing transformation (VST) normalized MGP expression levels extracted from the RNA sequencing dataset in preserved and lesioned OA cartilage stratified for rs1800801 genotype CC ($n_{Preserved}$=19 versus $n_{Lesioned}$=16) and CT+TT ($n_{Preserved}$=36 versus $n_{Lesioned}$=27). **[B]** Variance stabilizing transformation (VST) normalized *MGP* expression levels extracted from the RNA sequencing dataset in preserved and lesioned OA subchondral bone stratified for rs1800801 genotype CC ($n_{Preserved}$=5 versus $n_{Lesioned}$=5) and CT+TT ($n_{Preserved}$=9 versus $n_{Lesioned}$=9). The boxplots represent 25th, 50th and 75th percentiles, and whiskers extend to the 95%CI. Independent samples are depicted by black dots in each graph. To adjust for donor variation, P-values were estimated by performing logistic generalized estimation equations, with tissue status as dependent variable and *MGP* level, age and sex as covariate: *Tissue status ~ MGP level + age + sex + (1|Donor)*. * P≤0.05.

Next, we attempted to replicate the previously shown AEI of *MGP* in association with the OA risk SNP rs1800801 [7] in heterozygous individuals in this larger RNA sequencing dataset of preserved and lesioned OA cartilage [18] and a novel dataset of OA subchondral bone [19]. Additionally, we explored whether the effect size in AEI differed in these tissues between preserved and lesioned areas. As shown in **Supplementary Figure 2A** we confirmed AEI expression of *MGP* in preserved OA cartilage with the risk-conferring allele rs1800801-T associated to a reduced *MGP* expression of 10% (95%CI[2.24;18.64]) relative to the reference allele rs1800801-C. In lesioned OA cartilage the AEI was very comparable with rs1800801-T associated to a reduced *MGP* expression of 11% (95%CI[2.25;19.49]) relative to the reference allele rs1800801-C. In subchondral bone, genotype of rs1800801 could not be called thus we used its proxy SNP rs4236 (r^2=0.93 with rs1800801), which was also investigated previously [7]. As shown in **Supplementary Figure 2B**, we confirmed AEI of *MGP* in preserved OA subchondral bone with the risk-conferring allele rs4236-C associated to a reduced *MGP* expression of 10% (95%CI[5.70;14.52]) relative to the reference allele rs4236-T. In lesioned OA subchondral bone AEI was very comparable, with rs4236-C associated to a reduced *MGP* expression of 12% (95%CI[8.80;14.55]) relative to the reference allele rs4236-T.

Finally, we analysed *MGP* expression levels among genotype carriers of one or two of the OA risk alleles rs1800801-T (eQTL) and confirmed that also overall *MGP* expression in cartilage is reduced in a dose responsive manner with *MGP* risk alleles, independent of donor, age, sex, and OA status, i.e. preserved or lesioned (OR=0.73, 95%CI[0.64;0.84], P-value=$4.00x10^{-6}$; **Supplementary Figure 3A).** In subchondral bone we observed a similar pattern, however this was not significant (**Supplementary Figure 3B**). Together these data confirm that innate lower *MGP* expression levels confer risk to OA, though its effect seems more pronounced in articular cartilage.

MGP expression patterns in human in vitro and ex vivo models and as function of OA related cues

First, we investigated expression of *MGP* during neo-cartilage formation using a human *in vitro* 3D pellet culture with primary chondrocytes. As shown in **Figure 3**, *MGP* is expressed in primary chondrocytes (day-0) and increases during cartilage extracellular matrix (ECM) deposition until day-14, suggesting that *MGP* expression can be considered a marker of neo-cartilage formation.

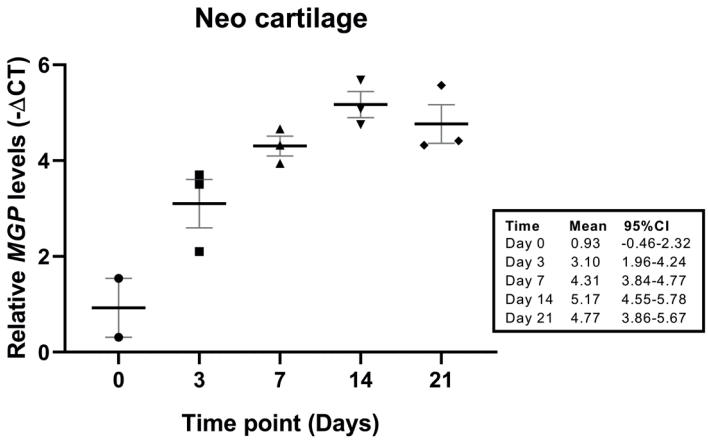

Figure 3 | Gene expression of MGP in an in vitro 3D model of neo-cartilage. Gene expression of MGP in an in vitro 3D chondrocyte pellet model of neo-cartilage formation (N=3 donors; day 0: n=2, all other time points: n=3). Data is depicted as mean expression (-ΔCT) ± standard error of the mean (SEM) and each dot represent an sample of two combined biological duplicates. Statistical analysis was performed by generalized linear mixed model (GLMM) using MGP levels as dependent variable and time as repeated measure: MGP level ~ Time + (1|Donor).

Next, we explored dynamic changes in *MGP* expression in cartilage and subchondral bone in an established human *ex vivo* osteochondral explant model [20] as function of OA related stimuli being inflammation (IL-1β), hypertrophy (T3), and 65% mechanical stress (65%MS). As shown in **Figure 4**, we observed in cartilage a consistent and significant downregulation of *MGP* expression after treatment with IL-1β (FC=0.03, 95%CI[0.02;0.06], P-value=$4.40x10^{-7}$), T3 (FC=0.80, 95%CI[0.56;0.97], P-value=0.046), as well as with mechanical stress (FC=0.65, 95%CI[0.45;0.85], P=0.002). Notable, in **Figure 4**, is an outlier

in the mechanical stress group, however removing this datapoint did not influence our result (FC=0.67, 95%CI[0.47;0.87], P-value=0.046). In subchondral bone we were not able to isolate RNA for IL-1β treated samples and only observed a significant downregulation of *MGP* expression after treatment with T3 (FC=0.81, 95%CI[0.52;1.10], P-value=0.015; **Figure 4 D-E**).

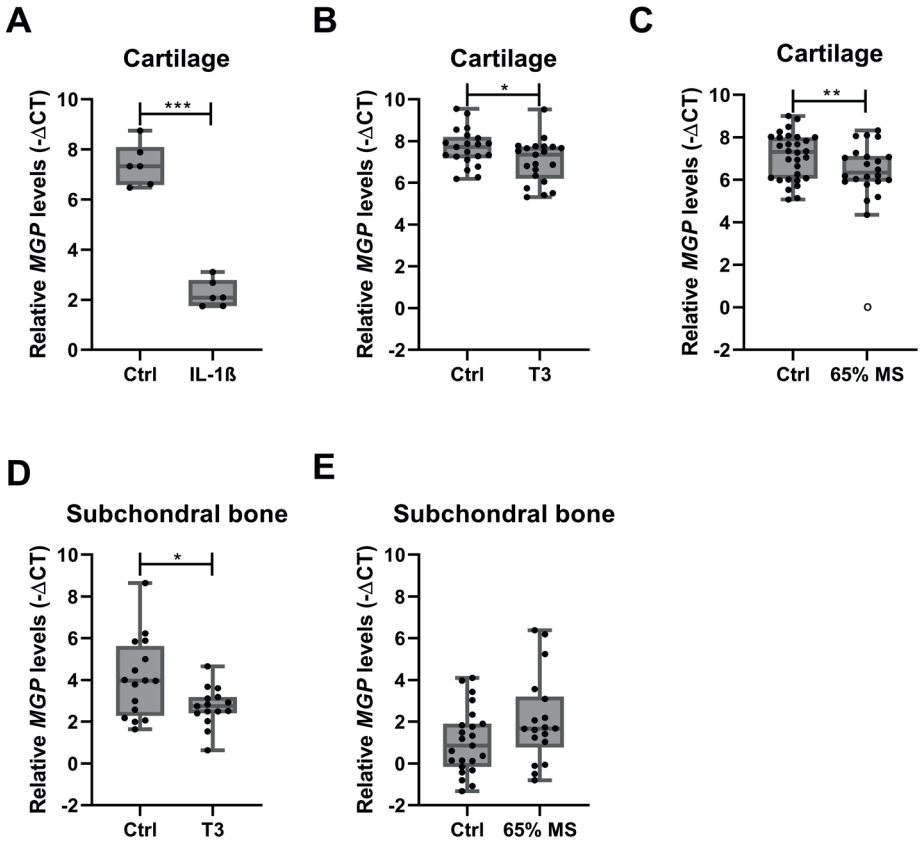

Figure 4 | Gene expression of MGP in response to three different OA relevant cues in cartilage of osteochondral explants. Gene expression of *MGP* (-ΔCT) in an *ex vivo* osteochondral explant model in articular cartilage (**A, B, C**) and subchondral bone (**D, E**). *MGP* expression, represented by the housekeeping gene corrected value (-ΔCT), in articular cartilage upon perturbation with **[A]** IL-1β ($n_{Control}$=6 versus $n_{treated}$=6), **[B]** T3 ($n_{Control}$=21 versus $n_{treated}$=21) and **[C]** posttraumatic OA after 65% MS ($n_{Control}$=30 versus $n_{treated}$=23). *MGP* expression in subchondral bone upon perturbation with **[D]** T3 ($n_{Control}$=16 versus $n_{treated}$=16) and **[E]** posttraumatic OA after 65% MS ($n_{Control}$=23 versus $n_{treated}$=19). The boxplots represent 25th, 50th and 75th percentiles, and whiskers extend to the 95%CI. -ΔCT of each independent sample is depicted by black dots in the graphs. To adjust for donor variation P-values were determined by performing linear generalized estimation equations, with *MGP* levels as dependent variable and treatment as factor: *MGP level ~ Treatment + (1|Donor)*. Far out values are represent by the white filled circle (o) which did not affect the result (see main body text) and therefore analysis including this sample is presented in **C**. * P≤0.05, **P≤0.01, *** P≤0.001. Legend: Ctrl=Control; 65% MS=65% mechanical stress.

Changes in MGP expression in the ex vivo OA models as function of the transcript and OA risk SNP rs1800801

Since general *MGP* expression was identified to change between preserved and lesioned OA cartilage and subchondral bone, and in a osteochondral explant model to several OA related stimuli (**Figure 4**), we next explored whether the OA risk allele rs1800801-T modified these effects. Hereto we investigated the observed dynamic downregulation of *MGP*, upon inducing hypertrophy (T3 exposure; **Figure 5A** and **C**) and mechanical stress (65%MS; **Figure 5B** and **D**) in our *ex vivo* cartilage explant model stratified by rs1800801 genotypes. For IL-1β treatment, donor numbers were too low to explore the effect of genotype.

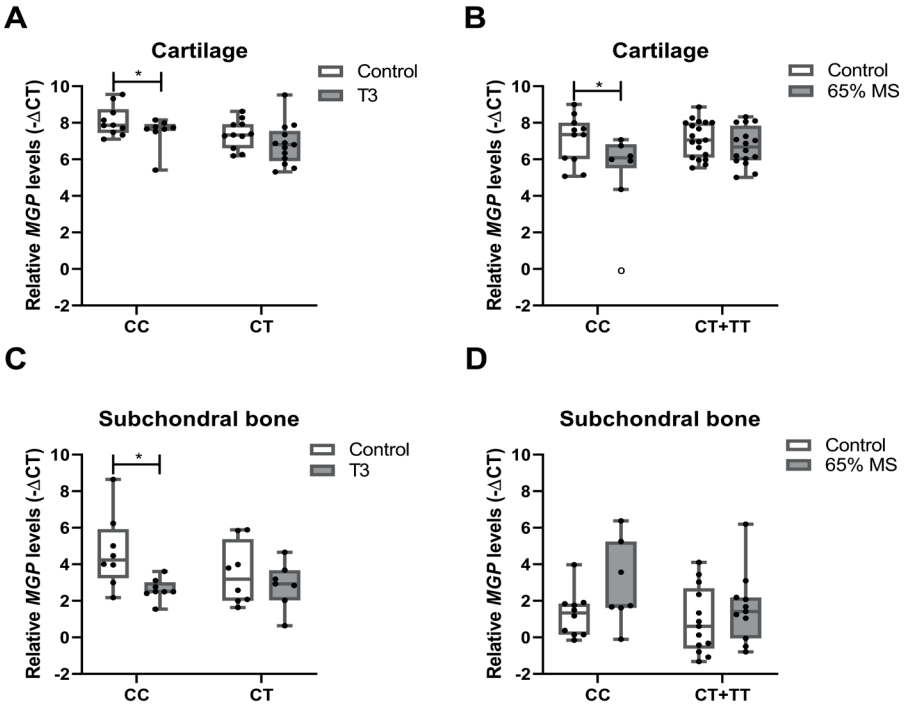

Figure 5 | *MGP* expression as function of the transcript and OA risk SNP rs1800801. *MGP* expression (-ΔCT) in an *ex vivo* osteochondral explant model stratified by rs1800801 genotype in articular cartilage (**A, B**) and subchondral bone (**C, D**). *MGP* gene expression in articular cartilage upon perturbation with **[A]** T3 (CC: $n_{Control}$=10 versus $n_{treated}$=8; CT: $n_{Control}$=11 versus $n_{treated}$=13) and **[B]** posttraumatic OA upon 65% mechanical stress (CC: $n_{Control}$=11 versus $n_{treated}$=7; CT+TT: $n_{Control}$=19 versus $n_{treated}$=16). *MGP* gene expression in subchondral bone upon perturbation with **[C]** T3 (CC: $n_{Control}$=8 versus $n_{treated}$=9; CT: $n_{Control}$=8 versus $n_{treated}$=7) and **[D]** posttraumatic OA upon 65% mechanical stress (CC: $n_{Control}$=10 versus $n_{treated}$=7; CT+TT: $n_{Control}$=13 versus $n_{treated}$=12). The boxplots represent 25th, 50th and 75th percentiles, and whiskers extend to the 95%CI. Independent samples are depicted by black dots in each graph. Numeric values associated to this Figure are shown in **Supplementary Table 4**. To adjust for donor variation P-values were determined by performing linear generalized estimation equations, with *MGP* levels as dependent variable and treatment as factor: *MGP level ~ Treatment + (1|Donor)*. Far out values are represent by the white filled circle (o) which did not affect the result (see main body text) and therefore analysis including this sample is presented in **B**. * P≤0.05. Legend: 65% MS=Mechanical stress.

In cartilage (**Figure 5A** and **Figure 5B**), we observed that downregulation of *MGP* occurred particularly among carriers of the reference allele rs1800801-C for hypertrophy (T3) (FC=0.69, 95%CI[0.49;0.89]) and for mechanical stress (FC=0.26, 95%CI[0.14;0.38]) as compared to carriers of the risk allele rs1800801-T for hypertrophy (FC=0.92, 95%CI[0.57;1.27]) and for mechanical stress (FC=0.85, 95%CI[0.61;1.09]). Also in the data shown in **Figure 5B** the previously identified outlier in the mechanical stress group did not influence our result upon removal (FC=0.29, 95%CI[0.17-0.41], P-value=0.045). As shown in **Supplementary Table 4**, the difference in response (FC) among carriers of the reference allele rs1800801-C relative to carriers of the OA risk allele rs1800801-T is significant for mechanical stress (FC=0.34, 95% CI[0.28-0.38], P-value=$2.8x10^{-3}$). Similarly in bone (**Figure 5C** and **5D**), we observed that downregulation of *MGP* expression in subchondral bone upon hypertrophy induction (T3) was more pronounced among carriers of the reference allele rs1800801-C (FC=0.50, 95%CI[0.09;0.91] as compared to the carriers of the risk allele rs1800801-T (FC=0.82, 95%CI[0.37;1.27]). This difference, however, did not reach statistical significance (**Supplementary Table 4**). For mechanical stress no effects were observed in subchondral bone. Together these data suggest that particularly in cartilage the OA risk allele rs1800801-T may have a different response in *MGP* expression upon OA relevant cues.

Treatment of osteochondral explants with warfarin

Since the activation of MGP is dependent on vitamin K and innate lower *MGP* expression confers risk to OA, we next investigated the direct effect of the vitamin K antagonist warfarin on articular chondrocyte and subchondral bone signalling. Hereto, *ex vivo* osteochondral explants (n=15 pairs for cartilage and n=13 pairs for subchondral bone) were treated with warfarin. The effect of this reduced vitamin K bioavailability on the cartilage homeostasis was determined by measuring chondroprotective genes (*SOX9*, *COL2A1* and *ACAN*), genes involved in early/late cartilage hypertrophy (*RUNX2*, *ALPL*, *COL1A1, COL10A1* and *MGP*) and catabolic genes (*MMP3*, *MMP13* and *ADAMTS5*). As shown in **Figure 6A**, warfarin exposure to cartilage reduced expression of *SOX9* (FC=0.87, 95%CI[0.77;0.97], P-value=0.023) and *MMP3* (FC=0.56, 95%CI[0.43;0.69], P-value=$1.02x10^{-5}$), while increasing *COL10A1* (FC=2.26, 95%CI[1.14;3.38], P-value=0.045). In addition, *RUNX2* (FC=1.43, 95%CI[0.96;1.90], P-value=0.094), a master transcriptional regulator of chondrocyte maturation, and *ALPL* (FC=6.21, 95%CI[1.36;11.06], P-value=0.059) show a trend towards upregulation in response to warfarin treatment. In subchondral bone, genes involved in matrix formation (*COL10A1, RUNX2*, *ALPL COL1A1, OMD, BGLAP* and *OGN*) and remodelling (*MGP, SPP1, TNFRSF11B, BMP2* and *POSTN)* were measured. As shown in **Figure 6B**, warfarin exposure to subchondral bone significantly reduced expression of the bone formation marker *COL1A1* (FC=0.81, 95%CI[0.59;1.03], P-value=0.046) and the remodelling marker *POSTN* (FC=0.67, 95%CI[0.42;0.92], P-value=0.011). Together these results show that addition of warfarin to aged osteochondral explants resulted in a significant upregulation of hypertrophic signalling among articular chondrocytes and reduced bone formation and altered remodelling signalling.

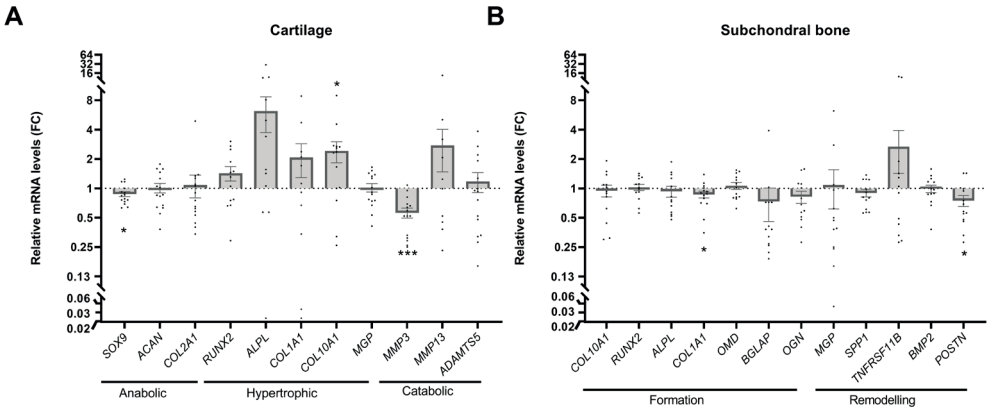

Figure 6 | Gene expression after 10 days of warfarin treatment. mRNA expression of genes depicted as a fold change (FC) following 50μM warfarin treatment relative to controls in **[A]** cartilage ($n_{Control}$=15 versus $n_{treated}$=15) and **[B]** subchondral bone ($n_{Control}$=13 versus $n_{treated}$=13). Controls are depicted by the dotted line, while each gray dot represents a warfarin treated sample. The number and percentage of missing data points per gene are summarized in **Supplementary Table 2** and **3**. The light gray bars represent the mean ± standard error of the mean (SEM) of the Fold change (FC) in which FC>1 represent upregulation and FC<1 represents downregulation of warfarin treated samples relative to its paired control. The x-axis is given in a log2 scale to depict the up and down regulation in the same scale. Differences in gene levels between warfarin exposure and controls were calculated by means of a paired t-test.*P ≤ 0.05; ***P ≤ 0.001

Discussion

In the current paper we explored (dynamic) changes of *MGP* expression in relation to the OA risk allele rs1800801-T, in preserved and lesioned OA cartilage, as well as, in a human *ex vivo* explant model subjected to OA related stimuli, such as inflammation, hypertrophy and mechanical stress. Furthermore, we studied the direct effect of the frequently used vitamin K antagonist, warfarin, on articular chondrocyte and subchondral bone signaling. In doing so, we confirm that *MGP* expression, as inhibitor of calcification via high affinity of calcium to its Gla-residues, should be considered a beneficial marker of articular cartilage. Consequently, the significantly upregulated *MGP* expression with ongoing OA pathophysiology is likely an attempt of chondrocytes to halt the OA associated osteo-induction. Noteworthy is our observation that the OA risk allele may also hamper adequate dynamic change in expression of *MGP* in response to OA and relevant cues like mechanical stress (65%MS) and this effect was most pronounced in cartilage. Finally, warfarin treatment to the aged human cartilage explants resulted in a significant upregulation of hypertrophic signalling among articular chondrocytes and reduced bone formation while altering remodelling.

Similar to previous reports [7,9], we here confirmed in a large RNA-sequencing dataset, that the OA risk allele rs1800801-T is associated with lower (overall) expression of *MGP* in articular cartilage (**Supplementary Figure 3A)**. In addition, we confirmed that *MGP* gene expression is significantly upregulated in both articular cartilage and subchondral bone in OA pathophysiology. Although, our results showed that this effect was mainly driven by carriers of the rs1800801-T OA risk allele in both tissues the expression of *MGP* does not reach the level of that in carriers of the reference allele rs1800801-C (**Figure 2A** and **2B**).

We advocate that *MGP* upregulation with OA pathophysiology in cartilage is an attempt of chondrocytes to compensate for the osteo-inductive effect of low *MGP* levels and that this is not sufficient among the *MGP* OA risk allele carriers. On the other hand, the upregulation of *MGP* in bone may be a marker of active bone resorption as it was previously found that MGP inhibits mineralization by osteoblasts while increased *MGP* expression in osteoclasts mark increased osteoclastic commitment [25]. Together our data highlights that, similar to vascular calcification and bone loss [26], also articular cartilage calcification and bone loss in OA could share a common pathogenetic mechanism involving MGP.

We also explored the dynamic response of *MGP* in a human *ex vivo* explant model while applying OA relevant perturbing cues such as inflammation, hypertrophy, and mechanical stress. The strength of our explant model is that it represents physiological relevant aged human articular cartilage prone to OA pathophysiology, hence suitable to study the initial process of OA related cartilage destruction. Moreover, and despite the inherent heterogeneity between donors, we found in cartilage a consistent downregulation of *MGP*, associated with matrix mineralization, as general response to OA related perturbations (**Figure 4**). Additionally, we showed that the rs1800801-T OA risk allele may hamper such innate dynamic change in *MGP* expression upon stress. A possible mechanism by which the genetic risk variant modifies response to stress lies in the fact that rs1800801 is localized in the transcription factor binding site (POLR2A, CTCF, p300) of the *MGP* promoter (**Supplementary Figure 4**). In addition, the OA risk allele rs1800801-T was shown to reduce expression between 34-47% in a luciferase reporter assay and *in silico* prediction suggested this to be due to a loss of binding site for the transcription factor c-Ets [12]. In the subchondral bone compartment of the human *ex vivo* explants, the *MGP* response to the OA like perturbing cues were smaller and less consistent although a similar *MGP* response appeared for T3 exposure. This is likely the result of (slightly) lower sample sizes but, more importantly, a more complex innate regulation and signalling of *MGP* in bone as multicellular tissue type. As such, the observed variation in the *MGP* response in bone remains inconclusive and needs to be repeated in larger sample sizes.

Upon identifying MGP, encoding an inhibitor of ectopic calcifications, as strong OA risk gene, it was hypothesized that the OA risk was conferred via calcification of cartilage tissue [10, 11]. Moreover, as MGP protein is activated by vitamin k dependent carboxylation (c-MGP) this finding underscored the relevance of previous found associations between OA and low vitamin K status [14,15]. Here, we showed that exposure of the vitamin k inhibitor warfarin to intact human articular cartilage explants provoked unbeneficial functional chondrocyte signaling towards hypertrophy, as reflected by upregulation of *COL10A1* and almost significant upregulation of *RUNX2* and *ALPL*. Moreover, we showed a modest but significant downregulation of *SOX9*, a transcription factor marking healthy articular cartilage. These observed effects of warfarin on chondrocyte signaling were similar to those previously found during *in vitro* knockdown of *MGP* in chondrocyte monolayer cultures [9]. With regard to the seemingly increased *MMP13* and reduced *MMP3* gene expression, it has been suggested that MMP3 plays a role mainly in healthy cartilage remodeling, while MMP13 more so in pathophysiological processes. This was confirmed by performing a look-up in our RNA-sequencing data set [18] were *MMP3* showed a marked downregulation in lesioned compared to preserved OA cartilage. Exposure of warfarin to subchondral bone of osteochondral explants

also provoked unbeneficial functional signaling towards reduced bone formation, as reflected by downregulation of *COL1A1* and the suggestive downregulation of *BGLAP,* whereas the upregulation of the osteoclastogenesis inhibitor *TNFRSF11B* (although not significant) and downregulation of the vitamin K dependent protein *POSTN* suggests altered bone remodeling likely resulting in bone loss [25,27]. Due to the low numbers and heterogeneity of patients, future studies are necessary to investigate if rs1800801 genotype influences response of cells to warfarin. In light of our result we advocate that the frequent prescription of warfarin as vitamin K dependent blood anticoagulant [16] may have clinical consequences in evoking OA comorbidity. As such, the risk of OA comorbidity may be considerably reduced by preferred prescription of non-vitamin K antagonist as anticoagulants [28]. In addition, vitamin K supplementation should be considered a potential novel OA-modifying treatment option. In this respect, there has been one underpowered clinical trial studying the effect of vitamin K supplementation on OA progression. This ancillary study, originally designed to study vascular calcification, reported no overall beneficial effects of vitamin K supplementation. However, in individuals with insufficient vitamin K levels at baseline a beneficial effect was observed [29].

Although the human aged macroscopically normal osteochondral explants used in our study may represent physiological relevant human articular cartilage and subchondral bone model, prone to OA pathophysiology, hence suitable to study the initial process of OA related destruction, the model is inherently subject to heterogeneity. Moreover, it does not provide insight into the *MGP* effect of such environmental perturbations to healthy cartilage and bone. Another limiting factor was the low sample size of T3, IL-1β and warfarin treated explants upon stratifying for rs1800801 genotype, resulting in no or less robust results than upon investigating the response in the larger mechanically stressed group. It should also be noted that the modifying effect of the *MGP* OA risk allele rs1800801-T as function of the OA status in articular cartilage and subchondral bone was only measured as a static effect i.e. differential expression of *MGP* between paired preserved and lesioned OA cartilage samples. Finally, the focus of our paper was on exploring gene expression changes of *MGP* only. Although studying protein levels of MGP as function of the OA risk SNP and the OA relevant cues in joint tissue would be an interesting addition and a preferred next step, such analyses should involve the detection of activated (hence carboxylated) MGP protein.

Together our data highlight that, similar to the bi-directional interplay of vascular calcification and bone loss in osteoporosis and atherosclerosis [26], also articular cartilage calcification and bone loss in OA might share a common pathogenetic mechanism likely involving MGP. Moreover, warfarin on human osteochondral explant cultures functionally underscores the previously found association between vitamin K deficiency and OA.

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Contributors

All authors have made contributions to the completion of this study. Study concept and design: EH, RCA, JM, YFM, IM. Acquisition of material and data: EH, MT, HED, DB, RGHHN. Data analysis: EH, RCA, MT, IM. Preparation of the manuscript: EH, IM. Critical reviewing and approval of the manuscript: All authors.

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Competing interests

None declared.

Patient consent for publication

Not required.

Data availability statement

Data are available on reasonable request.

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Supplementary Figure 4. Lookup of the rs1800801 SNP in USCS genome browser.

Supplementary methods

Neo-cartilage deposition

Primary chondrocytes were isolated from macroscopically preserved human articular cartilage and expanded as previously described [1] and a definition on what was determined as preserved and lesioned can be found in the first paper describing the Research in Articular Osteoarthritis Cartilage (RAAK) biobank [2]. *In vitro* 3D pellets were formed by centrifugation (1200 rpm, 5 minutes) using $2.5x10^5$ chondrocytes in 15 ml polypropylene conical tubes. Following maintenance in chondrogenic differentiation medium (CDM: DMEM (high glucose; Gibco, Bleiswijk), supplemented with Ascorbic acid (50 μg/ml; Sigma-Aldrich; Zwijndrecht, The Netherlands), L-Proline (40 μg/ml; Sigma-Aldrich), Sodium Pyruvate (100 μg/ml; Sigma-Aldrich), Dexamethasone (0.1 μM; Sigma-Aldrich), ITS+ and antibiotics (100 U/ml penicillin; 100 μg/ml streptomycin; Gibco)), while refreshing every 3-4 days, two pellets of each donor were pooled and harvested on day 0, 3, 7, 14 and 21 for RNA isolation. Experiments were repeated in two (day 0) to three (other time points) biological donors (N=3 donors).

DNA isolation and TaqMan genotyping

DNA was extracted from cartilage by pulverizing the tissue and homogenizing in Nuclei Lysis solution (Promega). DNA was extracted using the Wizard Genomic DNA purification kit (Promega) after overnight digestion using proteinase K (Qiagen) at 55°C. Conventional TaqMan genotyping was performed on genomic DNA using an allele-specific custom TaqMan assay for rs1800801 (Thermo Fisher Scientific) on a QuantStudio 6 Real-Time PCR system (Applied Biosystems).

RNA isolation

RNA was extracted from the cartilage and subchondral bone by pulverizing the tissue and homogenizing in TRIzol reagent (Invitrogen, San Diego, CA). RNA was extracted with chloroform, precipitated with ethanol and purified using the RNeasy Mini Kit (Qiagen, Chatsworth, CA). Genomic DNA was removed by DNase digestion and quantity of the RNA was assessed using a nanodrop spectrophotometer (Thermo Fischer Scientific Inc., Wilmington, USA). 200 ng of RNA was processed with the First Strand cDNA Synthesis Kit (Roche Applied Science, Almere, The Netherlands) according to the manufacturer's protocol. For several subchondral bone samples RNA isolation was not successful, reducing final sample size in the warfarin treated subchondral bone to 13 paired samples and for the other treatments as summarized in **Supplementary Figure 1B**. To exclude cross contamination of bone and cartilage samples, we measured gene expression differences of two cartilage specific genes (*COL2A1* and *COMP*) and two bone specific genes (*COL1A1* and *SPP1*) in 10 paired samples. As shown in **Supplementary Table 5**, we observe a relative high expression of cartilage markers and a low expression of bone markers in cartilage when compared to subchondral bone isolated from the same osteochondral explant. In subchondral bone we observed a relative low expression of cartilage markers and a high expression of bone markers, suggesting no to minimal cross contamination between cartilage and subchondral bone.

Reverse Transcription and Real-Time PCR

Real-Time PCR for gene expression was performed with QuantStudio 6 Real-Time PCR system (Applied Biosystems) using Fast Start Sybr Green Master mix (Roche Applied Science). Primer sequences used were tested for linear amplification and are listed in **Table 1**. Raw cycle threshold (CT) values for each sample were corrected for the average of one reference gene (*SDHA*) depicted as –ΔCt, and subsequently made relative to gene expression in controls (-ΔΔCt). Fold change (FC) was determined by calculating the log base 2 of the -ΔΔCt for each sample ($2^{-\Delta\Delta CT}$) where FC>1 is upregulation and a FC<1 is downregulation of a treated sample compared to the control sample of a donor. We used *SDHA* as reference gene since this gene was previously identified as a stable housekeeping gene and not responsive to mechanical stress in cartilage [3, 4]. In **Supplementary Table 2** and **3,** missing datapoints for genes measured by Real-Time PCR in cartilage and subchondral bone are summarized.

Unconfined dynamic (cyclic) compression of osteochondral explants

Explants were cultured in 24 wells plates (Greiner CELLSTAR; Sigma) supplemented with 1.5 ml CDM in a 5% (v/v) CO_2 incubator at 37°C. Six days after extraction, dynamic (cyclic) unconfined compression was applied to explant tissues using the Mach-1 mechanical testing system (Biomomentum Inc., Laval, QC, Canada) on four subsequent days **(Figure 1B)**. In short, osteochondral explant (diameter of 8mm) were placed under an indenter (diameter of 10mm) attached to a 250N MACH-1 load cell (**Figure 1C**) and unconfined cyclic compression was applied at a strain of 65% of cartilage height at a frequency of 1 Hz (1 compression cycle per second), mimicking walking speed, to give mechanical stress at stains suggested to be detrimental [5]. Due to the compression being unconfined, cartilage was allowed to displace sideway during compression. As shown in **Figure 1C**, dynamic (cyclic) compression means that a force was applied that varied over time to simulate a more cyclic compression such as walking. Media of explants was refreshed every three to four days. To investigate lasting effects of treatment, explants were harvested three days after the last treatment. Cartilage and bone were separated using a scalpel, snap-frozen in liquid nitrogen, and stored at -80°C for RNA isolation.

RNA sequencing data of cartilage and subchondral bone

In the current manuscript differential expression of *MGP* including false discovery rates (FDR) as multiple testing correction was taken from previously published transcriptome wide RNA-sequencing data of n=35 paired preserved and lesioned OA cartilage samples[6] and n=24 paired preserved and lesioned OA subchondral bone samples[7]. For $n_{cartilage}$=98 and $n_{subchondral\ bone}$=28 samples genotype of rs1800801 could be determined.

To test expression quantitative trait analyses (eQTL) and differential expression of *MGP* in genotype strata in the current manuscript, we used the variance stabilizing transformation (VST) normalized *MGP* expression levels of RNA sequencing datasets and used generalized estimating equations (GEE) [8] to effectively adjust for dependencies of genotypes among donors by adding a random effect for sample donor. For differential *MGP* expression analyses (**Figure 2**) a logistic model was applied with tissue status (preserved or lesioned) as

dependent variable and *MGP*, age and sex as covariates: *Tissue status ~ MGP level + age + sex + (1|Donor)* [9]. For genotype effects we show odds ratio's calculated from the exponent of the respective beta's. *MGP* expression quantitative trait loci (eQTL) analysis (**Supplementary Figure 3)** was performed by applying a linear GEE model with *MGP* level as dependent variable and dose response genotype, tissue status, age, and sex as covariate: *MGP level ~ rs1800801 genotype + tissue status + age + sex + (1|Donor).*

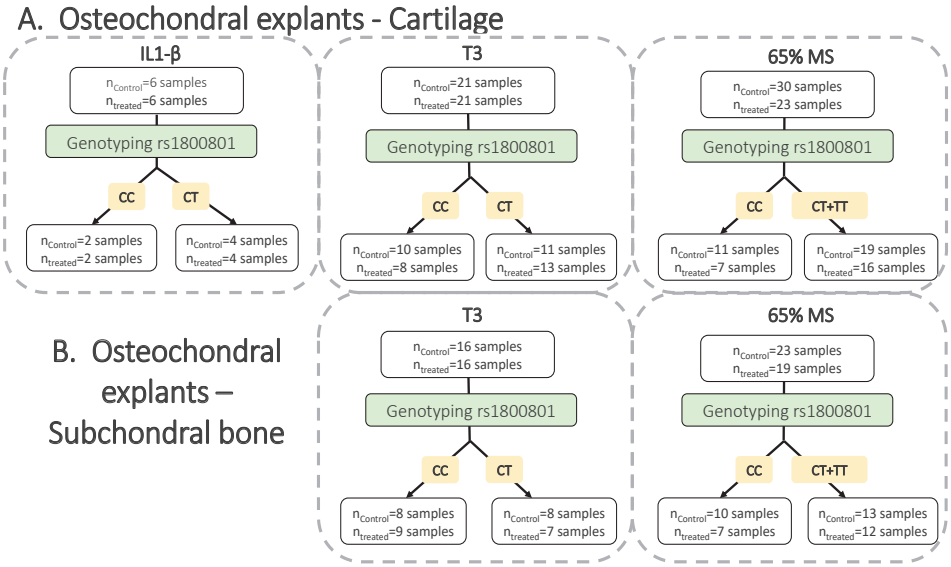
Allelic Expression Imbalance (AEI)

Allelic expression imbalance (AEI) was measured from the cartilage [6] and subchondral bone [7] RNA sequencing datasets for heterozygous individuals of rs1800801 in cartilage ($N_{preserved}$=37 and $N_{lesioned}$=28 samples) and rs4236 ($N_{preserved}$=12 and $N_{lesioned}$=12 samples) in subchondral bone as previously described in detail [10]. In short, reads of RNA-sequencing data were aligned using GSNAP against the hg19 reference genome, while potential reference alignment bias was masked using known Dutch SNPs (GoNL). Genotype of rs1800801 and other SNPs was called using SNVMix2 with default settings[11], with minimum coverage of 25 and at least 10 reads (**R**) per allele. Allelic imbalance is reported as the average fraction ($\boldsymbol{\varphi}$) of the alternative allele reads ($\mathbf{R}_{alternative}$) among the total number of reads ($\mathbf{R}_{total}=\mathbf{R}_{alternative}+\mathbf{R}_{reference}$) at the position of the respective genetic variation per sample ($\boldsymbol{i}$):

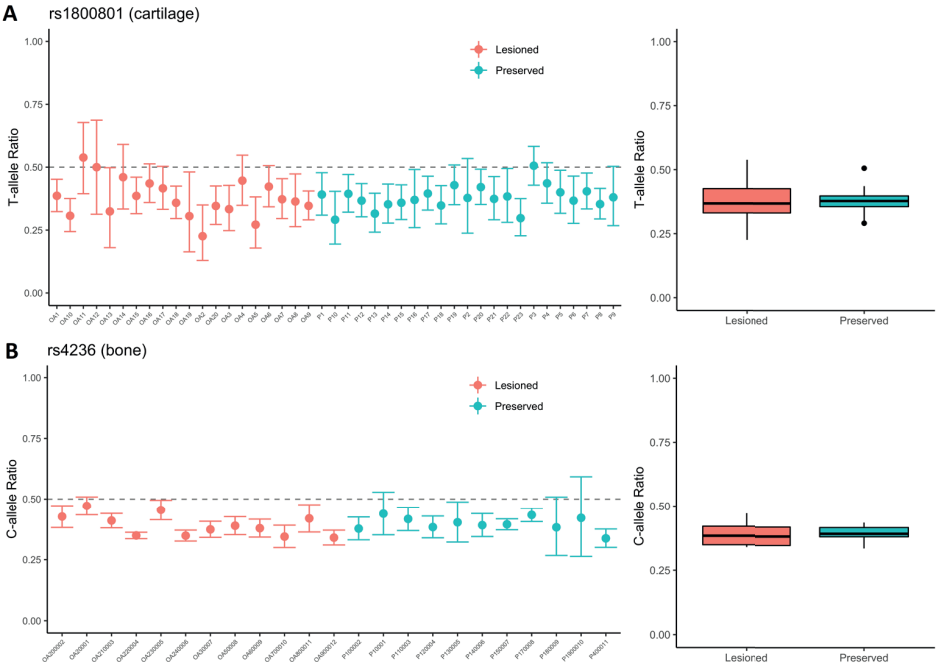
$$\boldsymbol{\varphi} = \frac{1}{n}\sum_{i=1}^{n}\frac{Ri, alternative}{Ri, reference}$$

To detect SNPs that robustly mark imbalance two binomial tests were performed per heterozygote and per SNP under the null hypothesis that the amount of imbalance is either greater or smaller than 0.49. Subsequently, P-values per SNP were corrected for multiple testing (FDR) by the number of heterozygotes of the respective SNP and considered significant if all FDR corrected P-values were <0.05 and in the same direction among all heterozygotes. Using the DEseq2 package, fragments per gene were used to assess the dispersion by quantile-adjusted conditional maximum likelihood (qCML)[12].

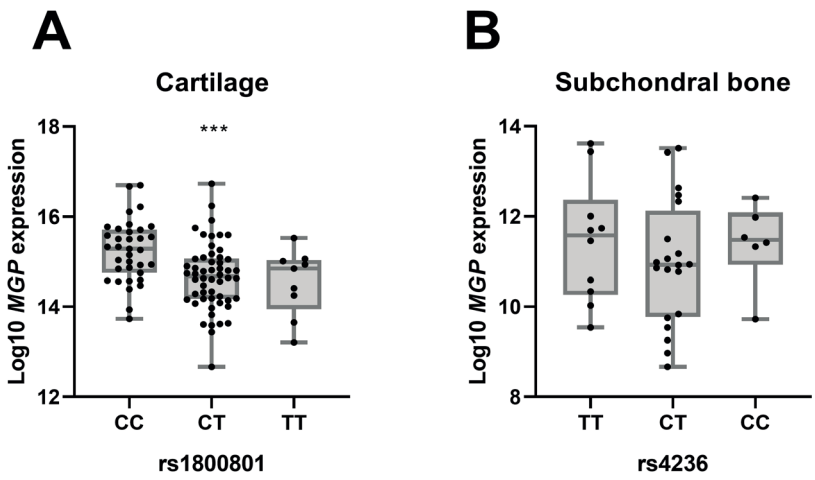
Supplementary Figures



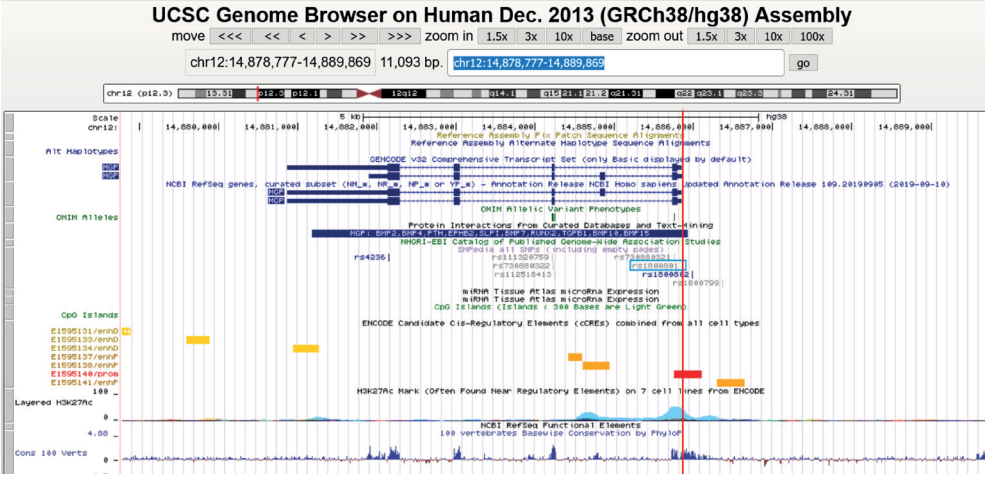
Supplementary Figure 1 | Schematic representation of the sample numbers used in the different osteochondral explant models. [A] Number of cartilage samples extracted from osteochondral explants for each condition (control and treated) and after splitting for rs1800801 genotype that were included for the different analysis on *MGP* expression. **[B]** Number of subchondral bone samples extracted from osteochondral explants for each condition (control and treated) and after splitting for rs1800801 genotype that were included for the different analysis on *MGP* expression. As we were not able to isolate RNA of all bone samples, numbers are lower when compared to cartilage.



Supplementary Figure 2 | Allelic expression imbalance of the OA risk allele rs1800801-T in articular cartilage and rs4236-C in subchondral bone. [A] The left part of the figure depicts the mean OA risk T-allele ratio with their 95%CI for the rs1800801 SNP in preserved and lesioned OA cartilage for each donor separate. The right part summarizes the T-allele ratio for independent lesioned (Red; N=28) and preserved (Blue; N=37) samples. A portion of this data was previously published by den Hollander W., et al [13]. **[B]** The left part of the figure depicts the mean OA risk C-allele ratio with their 95%CI for the proxy SNP rs4236 in preserved and lesioned OA subchondral bone for each donor separate. The right part summarizes the C-allele ratio for independent lesioned (Red; N=12) and preserved (Blue; N=12) samples. The dashed line at 0.50 represent the natural allele frequency when there is no allelic imbalance. The boxplots represent 25th, 50th and 75th percentiles, and whiskers extend to 1.5 times the interquartile range. Individual cartilage and bone samples are shown by random anonymised numbers.



Supplementary Figure 3 | Expression quantitative trait loci (eQTL) of MGP in articular cartilage and subchondral bone. [A] Variance Stabilizing Transformation (VST) normalized MGP expression levels extracted from the RNA sequencing dataset stratified for the OA risk SNP rs1800801 (CC: N=35; CT:N=54; TT: N=9) in preserved and lesioned OA cartilage. **[B]** Variance Stabilizing Transformation (VST) normalized MGP expression levels extracted from the RNA sequencing dataset stratified for a proxy (r^2=0.93 with rs1800801) of the OA risk SNP rs4236 (CC: N=10; CT:N=20; TT: N=6) in preserved and lesioned OA subchondral bone. The boxplots represent 25th, 50th and 75th percentiles, and whiskers extend to 1.5 times the interquartile range. Each samples is depicted by a black dot. Differences between groups was determined by measuring a dose response effect of the risk allele by performing generalized estimation equation (GEE) to correct for independency of genotype among the donors. The following linear GEE model was applied with MGP level as dependent variable and dose response genotype, tissue status, age, and sex as covariate: MGP level ~ rs1800801 genotype + tissue status + age + sex + (1|Donor). *** P≤0.001



Supplementary Figure 4 | Lookup of the rs1800801 SNP in USCS genome browser. The rs1800801 SNP (blue box) is found on chromosome 12 at nucleotide 14885854 (GRCh38/hg38) in the 5 Prime UTR of the *MGP* gene. The location of the SNP is depicted by the red line and is within the promotor region of the *MGP* gene. In addition, several transcription factor binding factors, such as PORL2A and CTCF are predicted to bind in this region (layer not shown).

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PEBP1
GGT7
MBIP
KAT2B
CKB
TET1
RCC2
TYRO3
LPIN1
CDKN1C
RCAN2
HTRA1
KLF2
SOCS3
IGFBP4
CD9
CDA
MYC
IGFBP6
STC2
FOXO1
GAPDH
ENG
IGFBP7
IGFBP5
SERPINE1
PRKAG2
NFKBIA
CAV1
PDK4
CYR61
EHD3
MMP13
FKBP5
FRZB
CMBL
TSC22D3
WARS
MXRA5
PSAT1
AMFR
EDEM1
GSN
SCUBE1
OS9
ALDH1L1
IARS
TNC
ITM2A
SLC25A5
ITGA10
RPL10A
TLN2
RPS12
RGCC
RIOK3
NEK6
DNHD1
CNTFR
CRLF1