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# Osteoarthritis and Cartilage

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# *In vivo* imaging of MMP-13 activity in the murine destabilised medial meniscus surgical model of osteoarthritis

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## ARTICLE INFO

## SUMMARY

*Objective:* To detect and determine disease severity of osteoarthritis (OA) using a probe activated by matrix metalloproteinase-13 (MMP-13) *in vivo* in the murine destabilised medial meniscus (DMM) surgical model of OA.

*Design:* We have previously described *MMP12ap* and *MMP13ap*, internally quenched fluorescent peptide substrate probes that are activated respectively by MMP-12 and MMP-13. Here we used these probes to follow enzyme activity *in vivo* in mice knees 4, 6 and 8 weeks following DMM surgery. After *in vivo* optical imaging, disease severity was determined through traditional histological analysis. The amount of probe activation was analysed for discrimination between DMM, contralateral and sham operated knees, as well as for congruence between activity and histological damage.

*Results:* There was no specific activation of *MMP12ap* at the time points observed between sham operated and DMM operated, or their respective contralateral joints. The activation of the *MMP13ap* in the DMM model was highest 6 weeks after surgery, but was only specific compared against sham surgery 8 weeks after surgery (1.5-fold increase). The activation of *MMP13ap* correlated with histological damage 6 and 8 weeks after surgery, with correlations of 0.484 ($P = 0.0032$) and 0.478 respectively ($P = 0.0049$). This correlation dropped to 0.218 ($P = 0.011$) if all data were considered.

*Conclusion:* The current MMP-13 activity probe is suitable for the discrimination between DMM and sham or contralateral knees 8 weeks after surgery, when cartilage loss is typified by the appearance of small fissures up to the tidemark, but not earlier. This activity correlates with the histological damage observed.

## Introduction

Osteoarthritis (OA) is a multifactorial disease, which leads to the destruction of the articular cartilage in joints and eventually the underlying subchondral bone. The search for an effective treatment of the disease is hampered by several factors. One of them is that disease is followed classically by radiographical measurement of joint space narrowing, which shows an advanced stage of tissue destruction where most of the articulating cartilage has been lost. To address this, increasing advances in magnetic resonance imaging (MRI) techniques have been applied to follow cartilage degradation in the clinic; this allows earlier prognosis[1,2]. It is, however, difficult to justify this expensive imaging modality in the pre-clinical development of drugs where a simple, but specific biochemical biomarker would suffice. However, there is still a lack of a robust biochemical biomarker for OA, with serum cartilage oligomeric protein levels being one of the most widely studied[3,4]. Other biomarkers such as the breakdown products following proteolysis of the major cartilage components, type II collagen and aggrecan, in blood and urine have also been examined[5]. There is a clear need of both biomarkers and non-invasive imaging methods of detecting early cartilage degradation for both drug discovery and diagnosis of OA.

The proteases responsible for the degradation of cartilage components are attractive targets for both biomarker and drug

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discovery, due to their established role in varied causes of OA. The major components of cartilage are the fibrillar type II collagen and the large aggregating proteoglycan, aggrecan. Members of the matrix metalloproteinase (MMP) and related adamalysin-like metalloproteinase with disintegrin and thrombospondin type I motifs (ADAMTS) family of proteases have been shown to be involved in this degradation[6]. In the destabilised medial meniscus (DMM) surgical model of OA, no degradation of aggrecan was observed in mice lacking functional ADAMTS-5 (agrecanase-2)[7], whereas no degradation of collagen was observed in mice lacking MMP-13 (collagenase-3)[8]. Mice lacking the protease cathepsin K also showed reduced cartilage damage in surgical models of OA[9]. However, it is still debatable whether this is a direct effect of cathepsin K degradation of collagen and aggrecan, or an indirect effect of cathepsin K upon the levels of ADAMTS-5 and MMP-13 in the joints of these mice. The ability to monitor the activity levels of these proteases during cartilage degradation may provide useful biomarkers of OA.

Advances in optical imaging technologies in conjunction with development of probes that are activated by proteases have enabled the detection of proteolysis *in vivo*[10]. Through the reverse design from MMP-specific inhibitors, we have developed substrate probes that are selectively cleaved by MMP-12 (macrophage elastase) and MMP-13 (collagenase 3)[11]. These peptide substrate probes are flanked by a near-infrared fluorophore and quencher such that upon cleavage, fluorescence can be detected at tissue transparent wavelengths. These activity probes, *MMP12ap* and *MMP13ap,* were used to study MMP-12 and MMP-13 activity in the development of arthritis in the collagen-induced model of murine rheumatoid arthritis. The probes showed that MMP-12 activity was increased early during inflammation where macrophage infiltration is observed whereas MMP-13 activity was detected later during joint destruction.

Here we test the use of the *MMP12ap* and *MMP13ap* to detect and follow OA disease progression in the DMM model of OA. Given the smaller role of inflammation in the DMM compared to collagen-induced arthritis, we hypothesised that there would be lesser activation of *MMP12ap* compared with to *MMP13ap*. If the MMP-13 activity probe was successful in detecting and following OA disease, it would be a useful tool during the development of disease modifying drugs and the early detection of OA.

## Methods

### Probes

The *MMP12ap* (QSY-21-GPLG ~ LEEAK(Cy5.5)G-OH) and *MMP13ap* (QSY-21-GGPAG ~ LYEK(Cy5.5)G-OH) probes were prepared and characterized as previously described[11].

### Animal models

Male C57 BL/6J mice aged 10 weeks were purchased from Harlan Laboratories (Blackthorn, Bicester, UK). Mice were housed in groups of 6 in individually vented cages, maintained at $21 \pm 2°C$ on a 12-h light/dark cycle and with food and water provided *ad libitum*. All experimental protocols were performed in compliance with the UK Animals (Scientific Procedures) Act 1986 regulations for the handling and use of laboratory animals (Home Office project licence PPL no: 70/6537).

The DMM surgery was performed on 10-week-old male mice as described previously[12]. Briefly, following induction of anaesthesia by injection of 10 μl/g of Hypnorm®/Hypnovel® (at a ratio of 1:1–4 parts water), the right knee joint was accessed via a medial incision. The right meniscotibial ligament was transected, resulting in the

release of the medial meniscus from its tibial attachment. Sham surgery consisted of medial capsulotomy of the right knee. All animals received buprenorphine HCl (Vetergesic; Alstoe Animal Health, York, UK) delivered sub-cutaneously immediately post surgery. A total of 82 animals were used (divided equally into DMM and sham surgeries, 36 for the *MMP12ap* probe and 46 for the *MMP13ap* probe). Recovery from surgery was monitored daily by weight, visual inspection of wound healing and behaviour for two weeks. There was no difference in weight gain between groups observed throughout the experiment.

### Activation of probes in vivo

Four, 6 and 8 weeks after surgery, 150 μl of 1 μM MMP-12ap or MMP-13ap was delivered intravenously via the tail vein. Two, 4 and 8 h after injection, consecutive fluorescence images were obtained by exposing mice under gaseous anaesthesia (2.5% (v/v) isoflurane and $O_2$) in a Kodak *In vivo* FX Pro (Carestream, Woodbridge, USA), to a 630 nm excitation and capturing emission at 700 nm for 1 min. X-ray images were taken at the same time point for co-registration of signal to the knee joint, by exposure to X-rays for 20 s.

### Image analysis

Fluorescence images were analysed using the Carestream MI software (version 5.1, Carestream, Woodbridge, USA). Regions of interest (ROI) were defined as a circle of 65 pixels for knees. The ROI for knees was centred in the joint space as visualised using co-registered X-ray images. The mean fluorescent intensity (MFI) of the ROI was then obtained. No background intensity was subtracted from the values.

### Histology and scoring of cartilage damage

After images at the 8 h time points were taken, mice were sacrificed by cervical dislocation and the knee joints were dissected and fixed in formalin (2% (v/v)) overnight, decalcified in ethylene diamine tetra acetic acid (EDTA) for 5 weeks and embedded in paraffin. Serial sections 5 μm in thickness were cut. Every tenth section was stained with Safranin O for scoring cartilage damage. Scoring of the extent of cartilage damage in individual sections was done in accordance with the guidelines laid out in the OARSI histopathology initiative for small animals on a scale from 1 to 6 by two different observers[13]. All four quadrants of the section (medial tibial plateau, lateral tibial plateau, medial femoral condyle, lateral femoral condyle) are scored individually and added for each histological section. The scores of the top three adjoining sections are added together to give the summed score (for a maximal score 72).

### Statistical analysis

All statistical analysis were performed with the software Prism (version 5.0, GraphPad Software, La Jolla, USA). Two way analysis of variance (ANOVA) with matched values and Bonferonni's post-tests were used to analyse the data in Figs. 3 and 4(A), paired Student's *t*-tests were used to analyse the data in Fig. 4(B) and Pearson's correlation without correction was used to analyse the data in Fig. 5(B)–(D).

## Results

### Progression of OA in the DMM model of OA

Histological analysis indicated that there was significantly increased damage observed in the DMM animals from 4 weeks
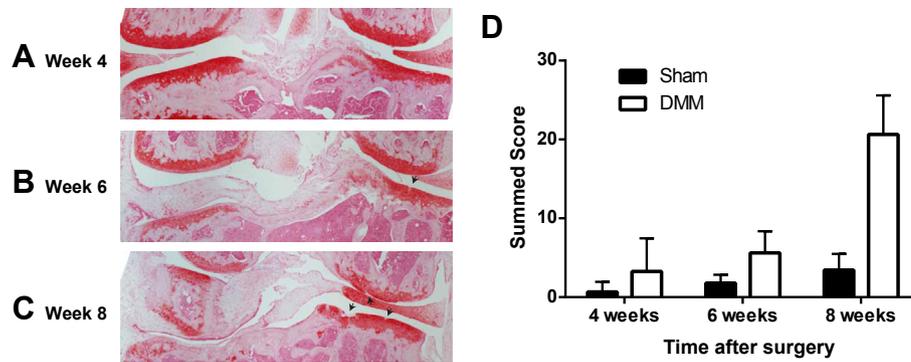
**Fig. 1.** Histological progression of DMM in this study. Typical histological sections of the DMM operated right knee joints stained with Safranin O obtained at (A) 4 weeks, (B) 6 weeks and (C) 8 weeks after surgery. Damaged areas within cartilage sections are indicated with arrows. (D) Summed histological scores obtained from addition of the scores of the top 3 consecutive scoring slides per knee. Data are mean ± 95% CI.

after surgery, which increased further at 6 and 8 weeks after surgery [Fig. 1]. These changes are comparable to those observed in DMM surgery performed elsewhere[12,14].

*Probes in the DMM model of OA*

Co-registered X-ray images were used to define the ROI of the knee joint in the fluorescent images. The 65-pixel circular ROI used is marked in the X-ray images in Fig. 2. Typical fluorescence images obtained using *MMP12ap* and *MMP13ap* are also shown in Fig. 2. The amount of signal obtained from the knees using both probes was between 200 and 2000 mean fluorescence units. This was lower than the signal which we obtained using the probes in collagen induced arthritis[11].

There was no significant difference in the activation of *MMP12ap* between DMM operated knees and control knees at any of the time points observed [Fig. 3, ($n$) = 6 in each group]. The non-disease-specific activation of *MMP12ap* was highest at 4 weeks and decreased as time progressed in all groups of animals. This

activation of *MMP12ap* is therefore most likely due to processes involved in the repair of the capsule of the knee joint in both DMM and sham surgeries.

Activation of *MMP13ap* was different from *MMP12ap* during the course of DMM. At the earliest time point, 4 weeks after surgery, there was no significant difference in probe activation between any of the groups at the 2, 4 and 8 h time points [Fig. 4(A), top]. This was also reflected when the signal difference between the operated and contralateral knee was calculated [Fig. 4(B), top]. Six weeks after surgery, there was significant difference in the amount of activated probe detected between the DMM operated and its contralateral knees at the 2 h time point according to two-way ANOVA, but no significant difference between DMM operated and sham operated knees at any of the other time points [Fig. 4(A), middle]. Furthermore, at 6 weeks, there was significant average difference of 400–600 units in the signal obtained from the DMM operated and its contralateral knees, whereas there was no difference between the sham operated and its contralateral knees [Fig. 4(B), middle]. Eight weeks after surgery there was significant difference between the
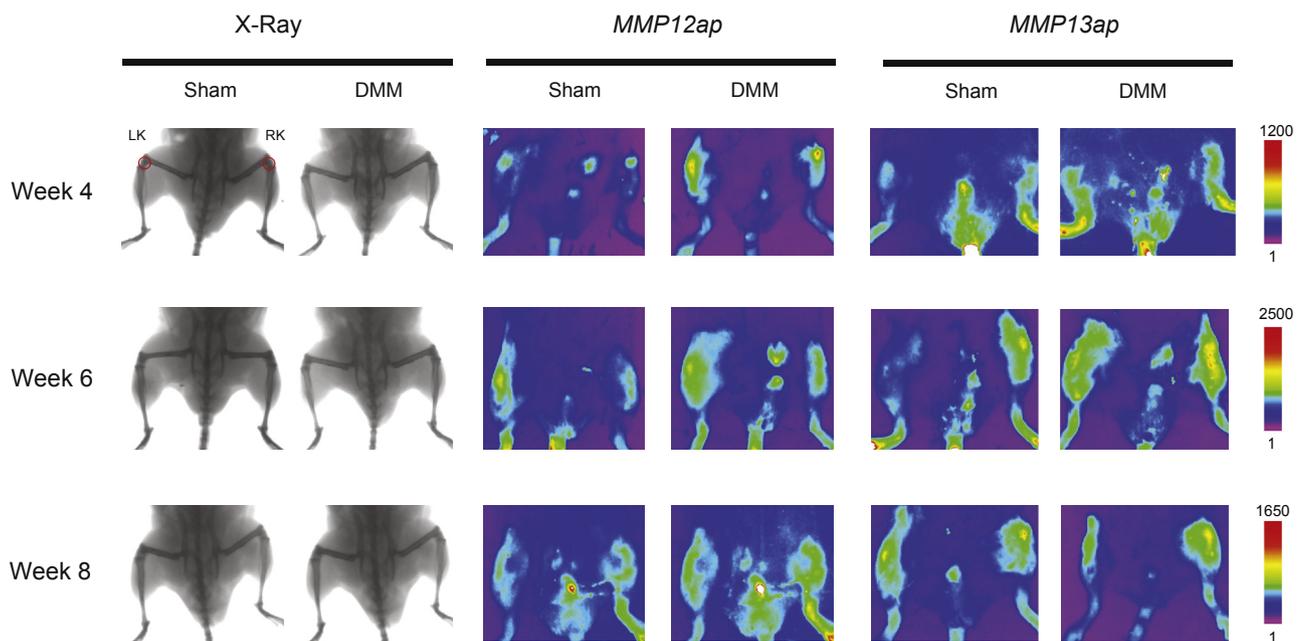


**Fig. 2.** Representative images obtained 4 h following intravenous injection of *MMP12ap* and *MMP13ap* in mice following sham and DMM surgery. Typical X-ray and fluorescence images obtained 4 h following intravenous injection of either 150 μl of 1 μM *MMP12ap* or *MMP13ap* after 4, 6 and 8 weeks after DMM or sham surgery. Left knees (LK), right knees (RK) and the red circle marking the ROI used for analysis are indicated. The fluorescence rainbow scales are indicated on the right.
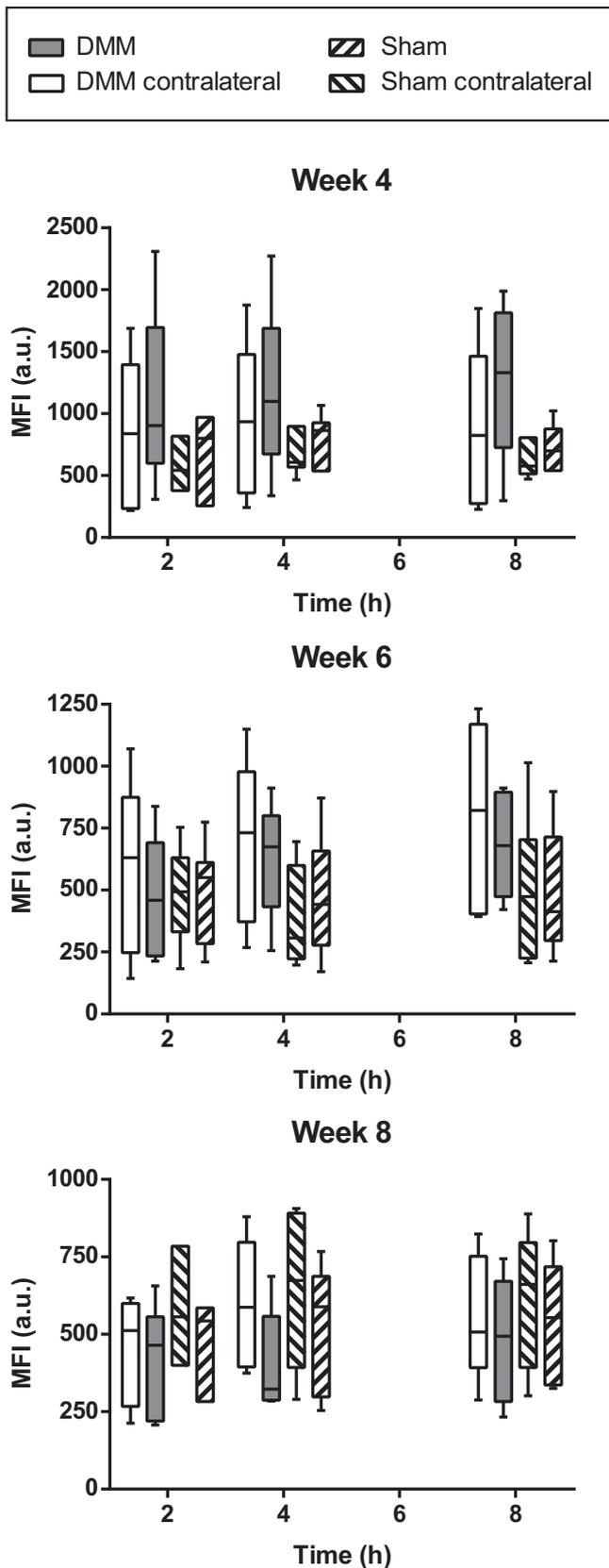
**Fig. 3.** Quantification of the activation of *MMP12ap* in the DMM model of OA. Mice were injected with 150 μl of 1 μM of *MM12ap* intravenously 4, 6 and 8 weeks after DMM or sham surgery. Images were taken 2, 4 and 8 h after injection and used to quantify the MFI in the operated and contralateral knees. Whiskers depict the 95% CI. *n* = 6 for each group. Analysis by two-way ANOVA with Bonferroni post-tests indicated no significant difference between any of the groups at any time points between the weeks.

signals obtained from the DMM operated knees compared with either the contralateral knee or the sham operated knees 4 h after the injection of probe [Fig. 4(A), bottom]. The significant average difference in signal between the operated and contralateral knees of the DMM operated animals was between 150 and 230 units throughout the time course [Fig. 4(B), bottom].

The earliest time at which *MMP13ap* could reliably distinguish the DMM operated knee from contralateral or sham operated knees was 8 weeks after surgery, with a 1.5-fold higher signal in the DMM knees compared to the controls. Earlier, at 6 weeks, the probe could differentiate between operated and contralateral knees only if it was known *a priori* that DMM surgery had been performed. The amount of *MMP13ap* activation detected was highest 6 weeks after surgery [Fig. 5(A)], indicating that the peak of detected MMP-13 activity occurred before the rise in histological damage. We have then examined whether there was any significant correlation between the activation of *MMP13ap* and histological damage in the knee joints, regardless if this damage was in the DMM, sham or contralateral groups. We found that there was no significant correlation between the activation of *MMP13ap* and histological damage 4 weeks after surgery [Fig. 5(B), $(r) = 0.26$, $P = 0.22$, $n = 24$]. However, there was significant correlation between activation of MMP13ap and histological damage separately at 6 and 8 weeks [Fig. 5(C) and (D)], with a correlation, r, of 0.484 ($P = 0.0032$, $n = 36$) and 0.478 ($P = 0.0049$, $n = 32$) respectively. In addition, when all the data was combined, there was weak but significant correlation between the activation of *MMP13ap* and histological disease score throughout the time period studied ($r = 0.263$, $P = 0.011$, $n = 92$). This indicates that histological tissue damage correlates with the activity of MMP-13 in the cartilage.

## Discussion

We have used two previously described activity probes for MMP-12 and MMP-13, *MMP12ap* and *MMP13ap* respectively, in the DMM model of murine OA to determine their utility in both disease diagnosis and disease severity. Inflammation plays a smaller role in the DMM model of OA, compared to the collagen-induced arthritis model of rheumatoid arthritis and this is reflected in the data obtained from *MMP12ap*. The lack of difference between the activation of *MMP12ap* in the DMM and sham operated groups of animals indicated there is no significant involvement of macrophage migration in the disease process at least at 4–8 weeks after the operation. The activation of *MMP13ap* however, discriminated between the DMM and controls 8 weeks after surgery, when cartilage damage is exemplified by small fissure(s) extending to the tidemark of calcified cartilage. This represents a relatively early stage in the degeneration of the type II collagen fibrils. The activation of *MMP13ap* also correlated with histological damage separately at 6 and 8 weeks after surgery, but this correlation weakened when combined, due to the higher levels of *MMP13ap* activation at 6 weeks. This indicates that there may be a peak in MMP-13 activity, and that damage to the tissue past a certain point is reliant on other factors, such as mechanical wear. This work represents the first in-depth study linking detectable *in vivo* MMP-13 activity from joints with OA disease progression.

While the *MMP13ap* probe is capable of differentiating between DMM and sham surgeries in this study, it is currently unsuitable as a biomarker for OA disease, owing to the weak overall correlation between probe activation and histological damage. Other studies using peptide substrate probes to detect MMP-13 activity in OA joints have focused on intra-articular delivery of the probe in rats[15]. Intra-articular delivery is suitable for studies using surgical models of OA, as the timing and joint location of disease occurrence is known. However, for models of spontaneous OA[16], such as those
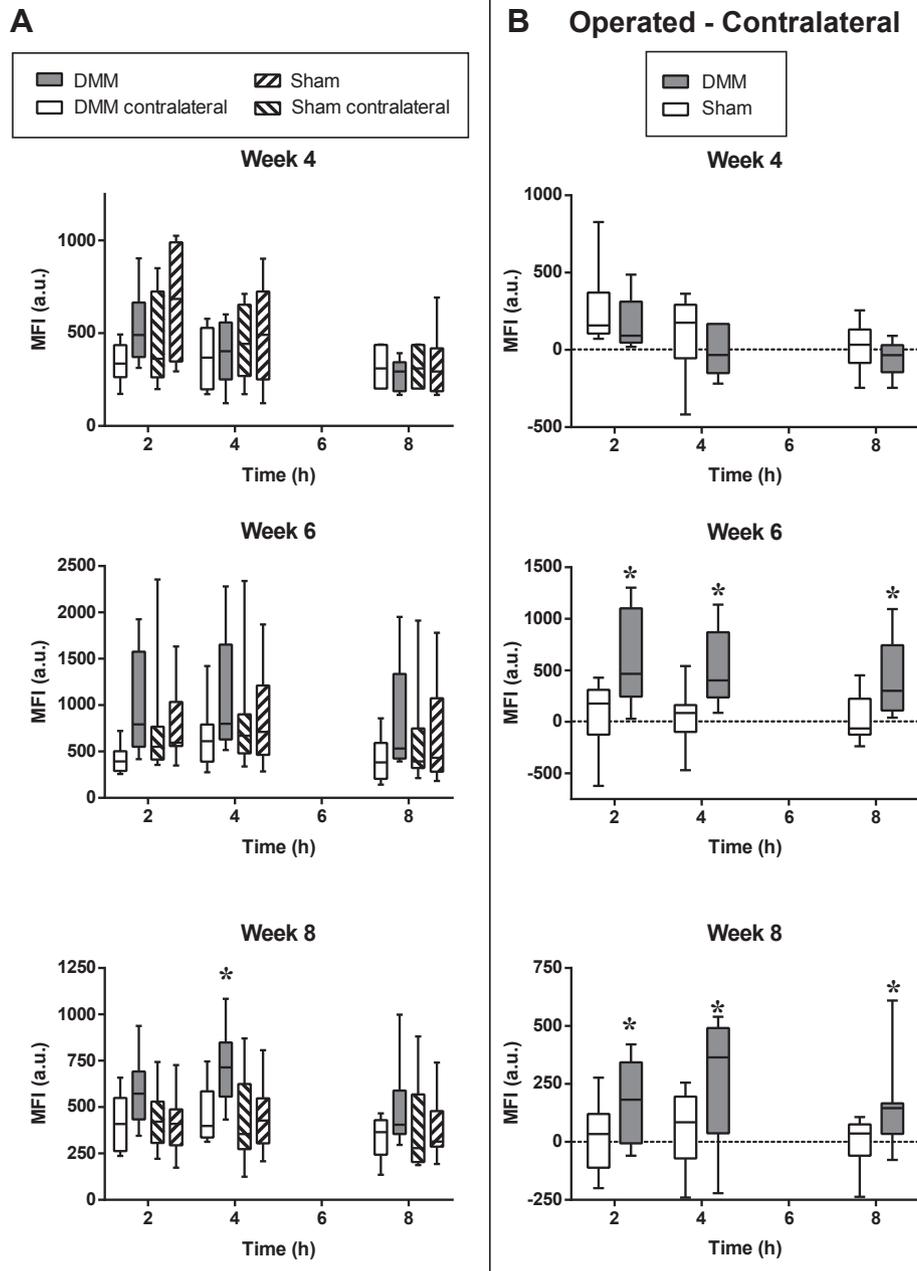
**Fig. 4.** Quantification of the activation of *MMP13ap* in the DMM model of OA mice were injected with 150 μl of 1 μM of the *MMP13ap* intravenously 4, 6 and 8 weeks after DMM or sham surgery. Images were taken 2, 4 and 8 h after injection and used to quantify the MFI in the operated and contralateral knees (A). Whiskers depict the 95% ci. $n = 6$ for each group in week 4, $n = 9$ for each group in week 6 and $n = 8$ for each group in week 8. *$P < 0.05$ between DMM and other groups after two-way ANOVA analysis with Bonferroni post tests (DMM vs contra: $P = 0.0477$, DMM vs Sham: $P = 0.0421$, DMM vs Sham contra: $P = 0.0161$). (B) The difference in signal between the operated and contralateral knees in the DMM and sham groups. *$P < 0.05$ by paired Student's t test, individual values as indicated.

involving the STR/ort mouse, or in larger animals such as the Duncan–Hartley guinea pigs, the ability to deliver probes intravenously becomes essential due to the unknown in both time and joint(s) affected. We have used this delivery route in this study with promising results. Improvements to the peptide probe described here, such as increased specificity, increased rate of cleavage by MMP-13 and increased retention in joint/cartilage, needs to be explored in order to realise its potential as a biomarker for OA disease progression.

Apart from mice deficient in MMP-13, mice deficient in ADAMTS-5 are protected in the DMM model[7,8]. An ADAMTS-5 activity probe may potentially serve as an earlier marker for OA, as aggrecan degradation is thought to occur before collagen

degradation[17]. There is, however, a higher turnover of aggrecan in normal tissue compared to collagen[18,19], which may make it difficult to differentiate the normal vs the pathological activity, limiting the utility of such an *in vivo* ADAMTS-5 probe.

Specific inhibitors for both ADAMTS-5 and MMP-13 are in development. Commonly used animal OA models in which the efficacies of these inhibitors are tested are those of the DMM or the anterior cruciate ligament injury model[20]. These models are well characterised in terms of where and when disease develops and how fast it progresses. Disease progression in human OA is currently unpredictable and the relative contributions of these proteolytic enzymes during this progression is not clear. Probes capable of detecting specific enzyme activity will allow us to start
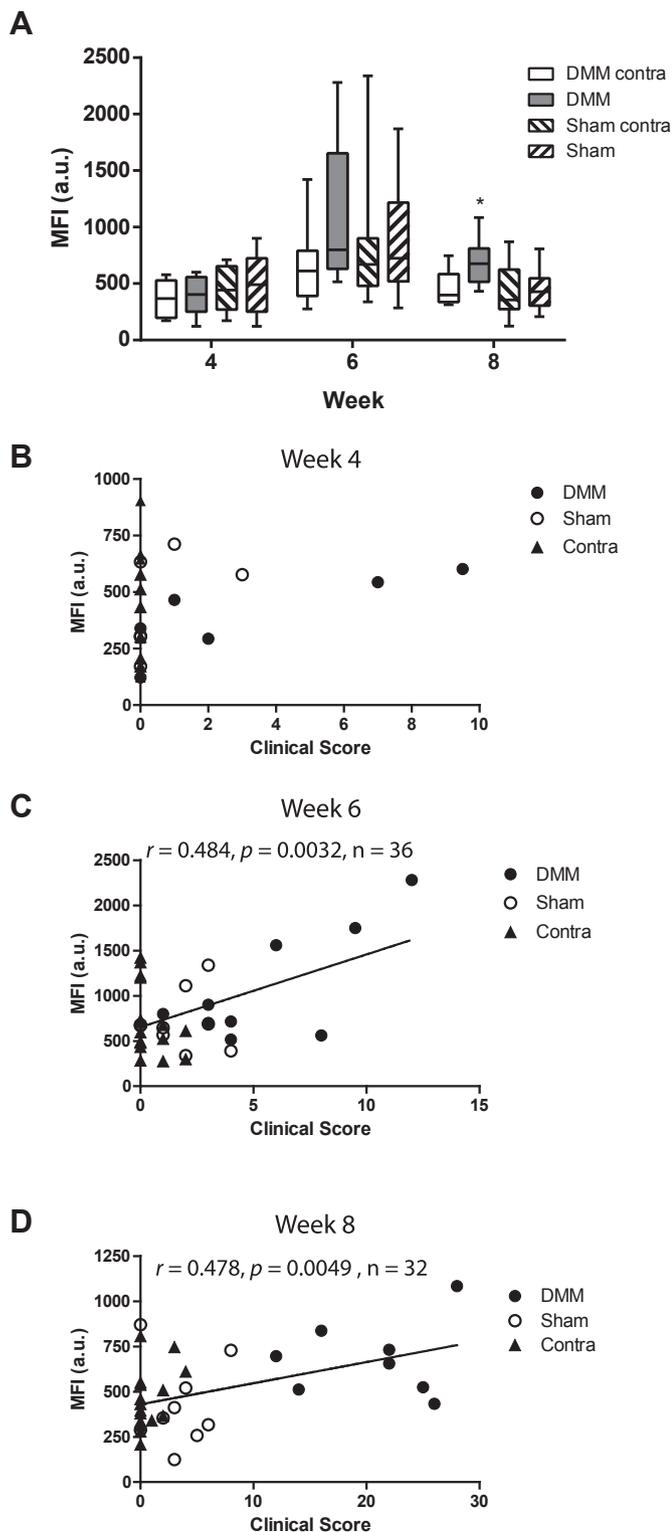
**Fig. 5.** Longitudinal correlation of the signal from the *MMP13ap* in the progression of the DMM model of OA The MFI of the relevant ROIs obtained at 4 h after injection of the *MMP13ap* was plotted against either the duration after surgery (A) or the individual summed histological scores 4 weeks (B), 6 weeks (C) or 8 weeks (D) after surgery. *$P < 0.05$ between DMM and other groups, as detailed in Fig. 4. The Pearson correlation factor, *r* at $P < 0.005$, between mean fluorescent signal and histological damage for weeks 6 and 8 are indicated.

to address this. Additionally, they may provide time frames where particular inhibitor therapies, should be prescribed, as there may be a window where an ADAMTS-5 inhibitor will be effective and a different window where an MMP-13 inhibitor would be. Thus, concurrent development of ADAMTS-5 and MMP-13 activity probes and their use may guide future management of clinical OA.

## Author contributions

Corresponding author Hideaki Nagase, George Bou-Gharios and Morten Meldal designed the project. Ngee Han Lim and Ernst Meinjohanns were involved with data acquisition and analysis, with George Bou-Gharios providing statistical expertise. Ngee Han Lim and Hideaki Nagase drafted the paper and all other authors reviewed the paper for important intellectual content. All the authors approved the final submitted version.

## Conflict of interest
There has been no commercial finance involved in this work and the authors declare no financial conflicts of interest.

## Role of funding source

## Acknowledgements

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