

Development of a translational strategy for using TIMP-3 to inhibit aggrecanase activity in osteoarthritis



Fabio A. Simoes^a, Greg Scutt^b, Manuela Mengozzi^a, Simon A. Mitchell^a, Jacob Keen^c, Katherine A. Staines^b, Linda Troeberg^d, Blandine Poulet^e, Andrew A. Pitsillides^c, Lisa M. Mullen^{a*}

^a Department of Clinical & Experimental Medicine, Brighton & Sussex Medical School, Brighton BN1 9PX, UK

^b Centre for Lifelong Health, School of Applied Sciences, Huxley Building, University of Brighton, Moulsecoomb, Brighton, UK

^c Comparative Biomedical Sciences, The Royal Veterinary College, Royal College Street, London, UK

^d Norwich Medical School, University of East Anglia, Norwich, UK

^e Institute of Lifecourse and Medical Sciences, University of Liverpool, Liverpool, UK

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ABSTRACT

Objective: Therapeutic potential of selective aggrecanase inhibition in osteoarthritis (OA) was previously demonstrated using a variant of endogenous tissue inhibitor of metalloproteinase-3 (TIMP-3); however, this relied on transgenic mice overexpressing TIMP-3. Here, we develop a translational approach for harnessing the aggrecanase-selective inhibitory activity of TIMP-3 using the latency associated peptide (LAP) technology. **Methods:** We successfully produced and purified recombinant LAP-TIMP-3 fusion proteins and determined the pharmacokinetics of these proteins *in vivo* following systemic injection. Surgical and non-surgical mouse models of OA were used to establish the therapeutic potential of these proteins in reducing aggrecanase activity in mouse joints affected by OA.

Results: The presence of the LAP conferred favourable TIMP-3 pharmacokinetics, with effective delivery of LAP-TIMP-3 to knee joints after systemic injection. We find that LAP-TIMP-3 also effectively reduced aggrecanase activity in OA-affected joints, both in spontaneously-occurring OA and in the destabilisation of the medial meniscus (DMM) model of OA. We also found that reductions in aggrecanase activity in articular cartilage correlated with improved disease scores, but only in earlier stages of disease.

Conclusions: This study describes the potential of LAP-TIMP-3 as a therapeutic agent in OA, showing delivery to the cartilage of joints affected by OA after systemic administration and lower levels of the neo-epitope of aggrecan in articular cartilage in mild disease (mean difference versus vehicle control for LAP-TIMP-3: 535 [95% CI: 336, 733] and for LAP-mutTIMP-3: 522 [95% CI: 323, 720] arbitrary units). These first *in vivo* data will inform further explorations into dose optimization and timing.

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Introduction

Hallmarks of osteoarthritis (OA) include destruction of articular cartilage extracellular matrix (ECM) and alterations in the underlying subchondral bone, both of which are enzyme-driven processes.

Many of the enzymes implicated in cartilage destruction in OA are members of the matrix metalloproteinase (MMP) family [1]. MMPs are upregulated in diseased or inflamed tissue, where repair or remodelling processes are essential [2]. MMP-13 in particular plays a significant role in type II collagen degradation resulting in cartilage fibrillation and loss of cartilage integrity in OA [3]. Aggrecanases are another key group of enzymes that contribute to pathological tissue remodelling [4]. In particular, ADAMTS-4 and -5 have emerged as key factors in OA [5–7] due to their proteolysis of aggrecan, a major component of articular cartilage [8].

Tissue inhibitor of metalloproteinases (TIMP)-3 is an important endogenous inhibitor of MMPs and aggrecanases [9,10]. Levels of TIMP-3 protein are reduced in cartilage of human OA joints [11],

* Corresponding author.

E-mail addresses: F.A.Simoes@bsms.ac.uk (F. Simoes), G.Scutt@brighton.ac.uk (G. Scutt), M.Mengozzi@bsms.ac.uk (M. Mengozzi), S.A.Mitchell@bsms.ac.uk (S. Mitchell), Jacob.Keen@soton.ac.uk (J. Keen), K.Staines@brighton.ac.uk (K. Staines), L.Troeberg@uea.ac.uk (L. Troeberg), b.poulet@liverpool.ac.uk (B. Poulet), apitsillides@rvc.ac.uk (A. Pitsillides), l.mullen@bsms.ac.uk (L. Mullen).

presumably resulting in reduced inhibition of MMPs and aggrecanases and thus contributing to cartilage degradation. This observation raised the possibility of using TIMP-3 as a therapy for OA. Indeed, there are many studies confirming its importance in maintaining a chondroprotective environment within the joint [12,13].

However, there are specific challenges associated with the utilization of TIMP-3 as a therapy, related to its biological functions and biochemical characteristics. Inhibition of MMPs using synthetic inhibitors were associated with numerous unacceptable side-effects [14,15], and since TIMP-3 inhibits a wide range of MMPs and aggrecanases, similar problems may arise. Studies in transgenic mice have elegantly demonstrated that both overexpression and deficiency of TIMP-3 have detrimental effects on bone mass and architecture [16,17]. Furthermore, TIMP-3 binds strongly to the ECM which makes it extremely difficult to purify in sufficient quantities for testing, either by expressing the recombinant protein or by purifying from tissues [10,18].

This ECM interaction also has a major impact on TIMP-3 half-life which, as elucidated by our previous work, is decreased by endocytosis via low-density lipoprotein receptor-related protein-1 (LRP-1) [19–21]. Mutants of TIMP-3 (e.g. K26A/K45A) exhibiting reduced affinity for LRP-1 and thus increased tissue half-life, show greater chondroprotective activity [13]. A further important finding was that addition of a single amino acid to the N-terminus of TIMP-3 ([–1A]TIMP-3) prevents its interaction with MMPs so that this version of TIMP-3 can selectively inhibit only aggrecanases and not MMPs [22]. The profound clinical implications of this narrowed specificity of [–1A]TIMP-3 is highlighted by studies showing that transgenic overexpression of [–1A]TIMP-3, rather than wild-type TIMP-3, resulted in cartilage protection in two different mouse models of OA [23,24]. This [–1A]TIMP-3 also produces increases in bone mass [16,17,23], an observation that is in stark contrast to the detrimental effects of wild-type TIMP-3 on bone [16,17].

As these previous studies relied upon the use of transgenic mice overexpressing [–1A]TIMP-3, we sought to take a more translational approach here, systemically administering exogenous recombinant versions of this molecule. We created a TIMP-3 molecule with the same specificity as [–1A]TIMP-3 using a drug delivery system known as the latency-associated peptide ('LAP') fusion protein technology [25–27]. This strategy is based on the naturally occurring LAP from the cytokine transforming growth factor (TGF)- β . Latent therapeutic agents are designed to be released from their protective LAP shell only at the sites of disease by the presence of a cleavage site specific for MMP located between the LAP and the therapeutic entity [27]. We applied the LAP technology to TIMP-3 by inserting a truncated MMP cleavage site in between the LAP and TIMP-3 that results in the addition of one amino acid (leucine) to the N-terminus of TIMP-3 upon cleavage of the molecule by MMP [25]. TIMP-3, when released from the LAP, can inhibit aggrecanase activity but no longer inhibits MMP activity, similar to that shown for [–1A]TIMP-3. The aims of this study were to: generate sufficient quantities of recombinant LAP-TIMP-3 with and without the K26A/K45A mutations for *in vivo* testing; determine the pharmacokinetics of these novel proteins; and for the first time in an *in vivo* setting, assess the dosing and timing necessary to inhibit aggrecanase activity in mouse joints affected by OA. We demonstrate favourable pharmacokinetics of the LAP-TIMP-3 proteins compared to TIMP-3 and, more importantly, show the potential of these proteins in inhibiting aggrecanase activity in joints affected by OA.

Materials and methods

Recombinant proteins

Three LAP-TIMP-3 proteins were produced for testing in mice: LAP-TIMP-3, LAP-mutTIMP-3 containing the TIMP-3 mutations

K26A/K45A and LAP-TIMP-3 No-CLV, a molecule lacking the MMP cleavage site between the LAP and TIMP-3. These proteins were expressed and purified by Icosagen Cell Factory (Estonia) as described in [Supplementary Methods](#) and [Supplementary Figure 1a](#). TIMP-3 was released from the LAP dimer by treatment with MMP-1 *in vitro*, whereas LAP-TIMP-3 No-CLV remained intact upon incubation with MMP-1 ([Supplementary Fig. 1b](#)). Attempts were made to express and purify L-TIMP-3 (i.e. TIMP-3 with a N-terminal leucine and without the LAP). However, it was not possible to purify this protein in any appreciable quantity, similar to previous reports for native TIMP-3 in the absence of the LAP [18,25]. Therefore, small quantities of untagged recombinant TIMP-3, without the LAP, was purchased from R&D Systems (Abingdon, Oxon, UK) for use as a control in pharmacokinetic experiments.

Animals and *in vivo* studies

Animal work was approved by the Home Office and conducted in accordance with the Animals (Scientific Procedures) Act 1986. All mice were housed on a 12-hour light/dark cycle with unrestricted access to food and water. C57BL/6 male mice were purchased from Charles River and housed in cages of 5 animals at the University of Sussex. STR/Ort mice [28,29] from a colony maintained at the Royal Veterinary College, London were used at 18 weeks of age. Male mice were used in all experiments as female mice show less joint degeneration in these models [30,31]. Animals were randomly assigned to treatment groups in a randomised block design and analyses were conducted blindly to minimise the effects of subjective bias. Animal studies were conducted and reported in line with the ARRIVE guidelines.

Pharmacokinetic studies

Twelve-week-old C57BL/6 mice were injected with purified recombinant proteins and blood samples obtained from the tail vein at 15, 30, 60, 120, 180 and 240 min for LAP-TIMP-3 proteins and at 5, 15, 30, 45, 60 and 120 min for TIMP-3. Mice were sacrificed within 24 h by overdose of pentobarbital and tissues collected by snap-freezing in liquid nitrogen or fixation in 4% neutral buffered formalin for hind limbs. Blood samples were allowed to clot for 24 h before centrifuging to pellet clotted material. Serum was collected and stored at –80 °C until further use.

In vivo loading

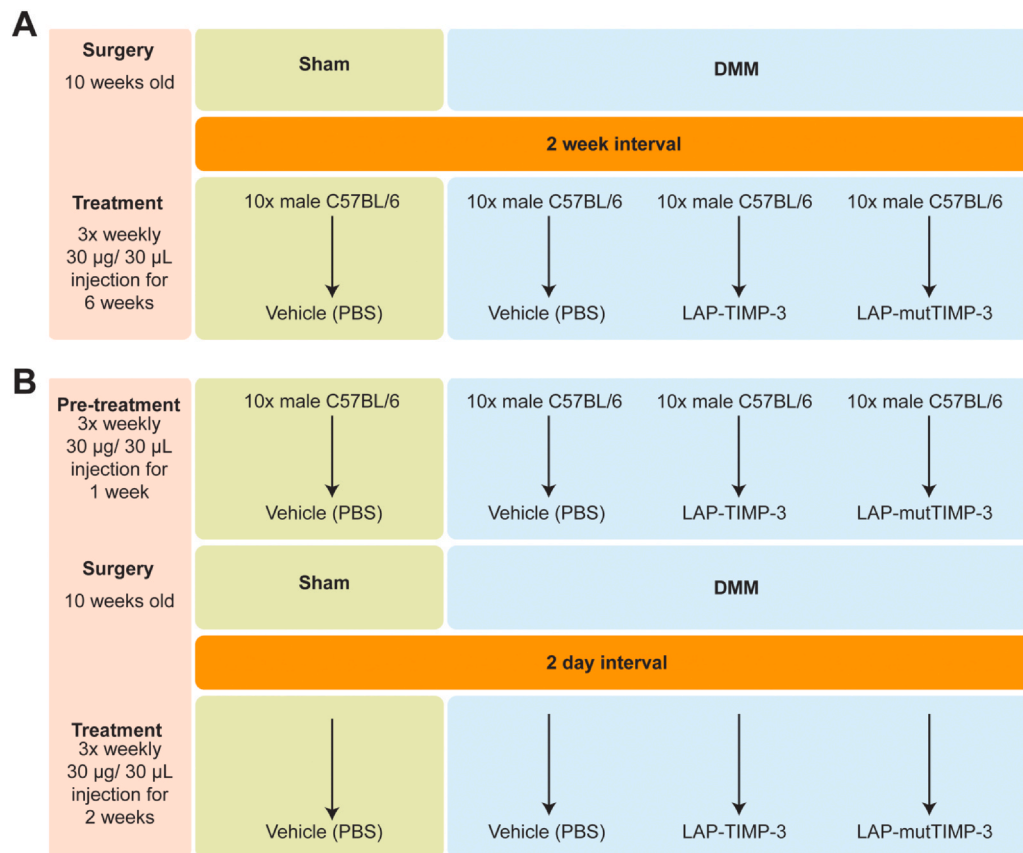
The right knee joints of 18-week-old male STR/Ort mice were subjected to a previously described joint loading regimen in which the right knee was subjected to three precisely controlled loading episodes each week for two weeks [32].

Surgical joint destabilization

10-week-old C57BL/6 underwent surgery to destabilise the medial meniscus (DMM), or sham surgery of the knee joint as previously described [33,34]. Briefly, animals were placed under general anaesthesia using isoflurane and microsurgery performed to transect the meniscotibial ligament using a surgical microscope. For sham operations, the knee joint was opened, the meniscotibial ligament identified but not transected and the incision closed with sutures.

Treatment with recombinant proteins

Two treatment regimens were tested using the DMM model of OA with each mouse designated an experimental unit ([Fig. 1](#)). STR/Ort mice were treated with 1 mg/kg of protein 3 times per week for 3 weeks starting at 18 weeks of age. Mice were sacrificed at the end of each experiment by overdose of pentobarbital and blood and tissue samples collected in RNAlater reagent (Invitrogen, UK) or snap frozen in liquid nitrogen.

**Fig. 1**

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Experimental design for testing effects of LAP-TIMP-3/LAP-mutTIMP-3 proteins on disease induced by DMM surgery in C57BL/6 mice. A. Protocol for testing the effects of LAP-TIMP-3 proteins on relatively severe disease, where treatment commenced two weeks after DMM surgery and continued for 6 weeks. B. Protocol for testing the effects of the recombinant proteins on milder disease where mice were treated for 1 week prior to surgery and for two weeks after surgery. Sample sizes were determined by a priori power calculation using OARSI disease scores as the primary outcome measure.

ELISAs

Various ELISAs were used to measure the concentrations of LAP-TIMP-3 proteins in mouse sera or tissue homogenates as described in [Supplementary Methods](#). All samples were measured in triplicate and optical densities determined using a spectrophotometric ELISA plate reader (BioTek, Swindon, UK) and analyzed using Gen5 software V2.6.

Histology and disease scoring

Knee joints were collected for histological analyses as described in [Supplementary Methods](#) using the whole joint Osteoarthritis Research Society International (OARSI) scoring system [35] or a more granular scoring system as detailed in [Supplementary Table 1](#) to assess very mild disease.

Immunohistochemistry

Knee sections were prepared for immunohistochemistry and labelled using anti-TIMP-3 or anti-NVTEGE antibodies as described in the [Supplementary Methods](#).

Pharmacokinetic analysis

Two-compartment pharmacokinetic parameters were determined using previously described methodologies [36,37] as described in the [Supplementary Methods](#).

Statistical analyses

Data are expressed as the mean $\pm$ SD, with mean differences and 95% confidence intervals for statistically significant results, and were analyzed using GraphPad Prism (GraphPad Software). All means are unadjusted unless otherwise stated. For comparisons of 3 or 4 groups, a one-way analysis of variance (ANOVA) or two-way ANOVA with Tukey's multiple comparisons test was applied for normally distributed data. For not normally distributed data, Kruskal-Wallis test followed by Dunn's multiple comparison test was applied. For normally distributed data with unequal SDs, Brown-Forsythe ANOVA followed by Dunnett's multiple comparison test was applied. All data points were included in the data analysis except for [Fig. 7a](#), where an outlier was identified using the ROUT method prior to analysis.

Results

LAP improves the pharmacokinetics of LAP-TIMP-3/LAP-mutTIMP-3 compared to TIMP-3

To investigate *in vivo* pharmacokinetics LAP-TIMP-3, LAP-mutTIMP-3 or TIMP-3 were injected into mice via the tail vein and blood samples collected over time. A concentration of 1 mg/kg was used for these experiments to ensure that there would be sufficient quantities of the recombinant proteins in the blood to measure by ELISA. The concentrations of the proteins in the sera were measured by LAP or TIMP-3 ELISA (Fig. 2a). Data collected from mice injected with recombinant proteins was used to estimate pharmacokinetic parameters for the three TIMP-3 formulations; a two-compartment pharmacokinetic model (Supplementary Fig. 2) provided a more accurate description of our experimental data than a single compartment model. A summary of all two-compartment pharmacokinetic parameters is shown in Table 1. Compared to TIMP-3, the presence of the LAP reduced mean clearance by approximately 75% (CI, Table 1, Fig. 2b, $p < 0.0001$; mean difference of 3.2 mL/min [95% CI: 2.5, 3.9] for LAP-TIMP-3 and of 3.4 mL/min [95% CI: 2.7, 4.1] for LAP-mutTIMP-3 versus TIMP-3), with the apparent volume of distribution of the central compartment (V_c) remaining similar for both formulations. Consequently, there is a significantly lower elimination rate constant (k_{10} , Figure 2c, Table 1, $p < 0.0001$ mean difference of -0.11 min^{-1} [95% CI: -0.14 , -0.08] for LAP-TIMP-3 and of -0.097 min^{-1} [95% CI: -0.13 , -0.07] for LAP-mutTIMP-3) for LAP-TIMP-3/LAP-mutTIMP-3 compared to TIMP-3.

The distribution rate constant (α) was also significantly decreased by the presence of LAP, indicating altered entry and exit kinetics. The complex changes to the distribution kinetics and elimination rate constants leads to a significant increase in both the total and central Mean Residence Time (MRTc) for LAP-TIMP-3/LAP-mutTIMP-3 and a small, but statistically insignificant increase in the peripheral Mean Residence Time (MRTp) (Table 1). Apparent volume of distribution at steady state (V_{ss}) and the apparent peripheral volume of distribution (V_p) were also found to be different for the LAP and non-LAP TIMP-3 formulations (Fig. 2d and e). A simulation of central (plasma) and peripheral concentrations as a function of time using the experimentally derived model parameters described in Table 1 is shown in Fig. 2f. The simulation illustrates that the smaller k_{10} and k_{12} values for the LAP formulations produces elevated plasma (central) concentrations compared to non-LAP TIMP-3 over a 200 min duration.

We also repeated these experiments using intraperitoneal injections to determine whether the route of systemic administration affected entry of the proteins into the circulation (Fig. 2g). As expected, an initial rapid absorption phase was observed in the samples collected from mice injected via intraperitoneal injection, with all three proteins entering the circulation. The relative bioavailability of intraperitoneal vs. intravenous LAP-TIMP-3 was $F_{rel}=90\%$. Non-LAP TIMP-3 was again cleared from the blood more quickly than the LAP-TIMP-3 proteins and the absorption rate was also faster than for the LAP fusion proteins after intraperitoneal injection (Fig. 2g).

LAP-TIMP-3 is detectable in knee joints after intraperitoneal administration

To determine whether concentrations of LAP-TIMP-3 in the blood, tissues and knee joints were dose dependent after systemic injection, we employed LAP-TIMP-3 No-CLV protein injections. We found that there was a dose-dependent increase in the concentration of LAP-TIMP-3 No-CLV in the serum at all time points measured, with the higher doses of protein remaining in the circulation for longer

(Fig. 3a). A steady-state concentration curve (Fig. 3b) showed deviation from linearity between 5 and 10 mg/kg, indicating saturation of elimination pathways between these two doses.

Importantly, upon 1 mg/kg injection, we could also detect LAP-TIMP-3 No-CLV in the knee joints of mice by immunolabeling with anti-TIMP-3 antibody. There was very little native TIMP-3 detected in the joints of un-injected mice but TIMP-3 was detected in the chondrocytes of the articular cartilage in the knees of mice injected with LAP-TIMP-3 No-CLV at 4, 8 and 24 h after injection (Fig. 3c). These data indicate that formulations of the LAP-TIMP-3 proteins delivered by intraperitoneal injection can circulate systemically and are present in knee joints for at least 24 h. To discern whether certain tissues could act as reservoirs for the proteins, several tissues were collected from mice 4, 8 or 24 h after intraperitoneal injection of varying concentrations of LAP-TIMP-3 No-CLV, and protein levels evaluated in tissue homogenates by His ELISA (Supplementary Fig. 3). There was no indication that the recombinant protein remained in any of these tissues 24 h after injection.

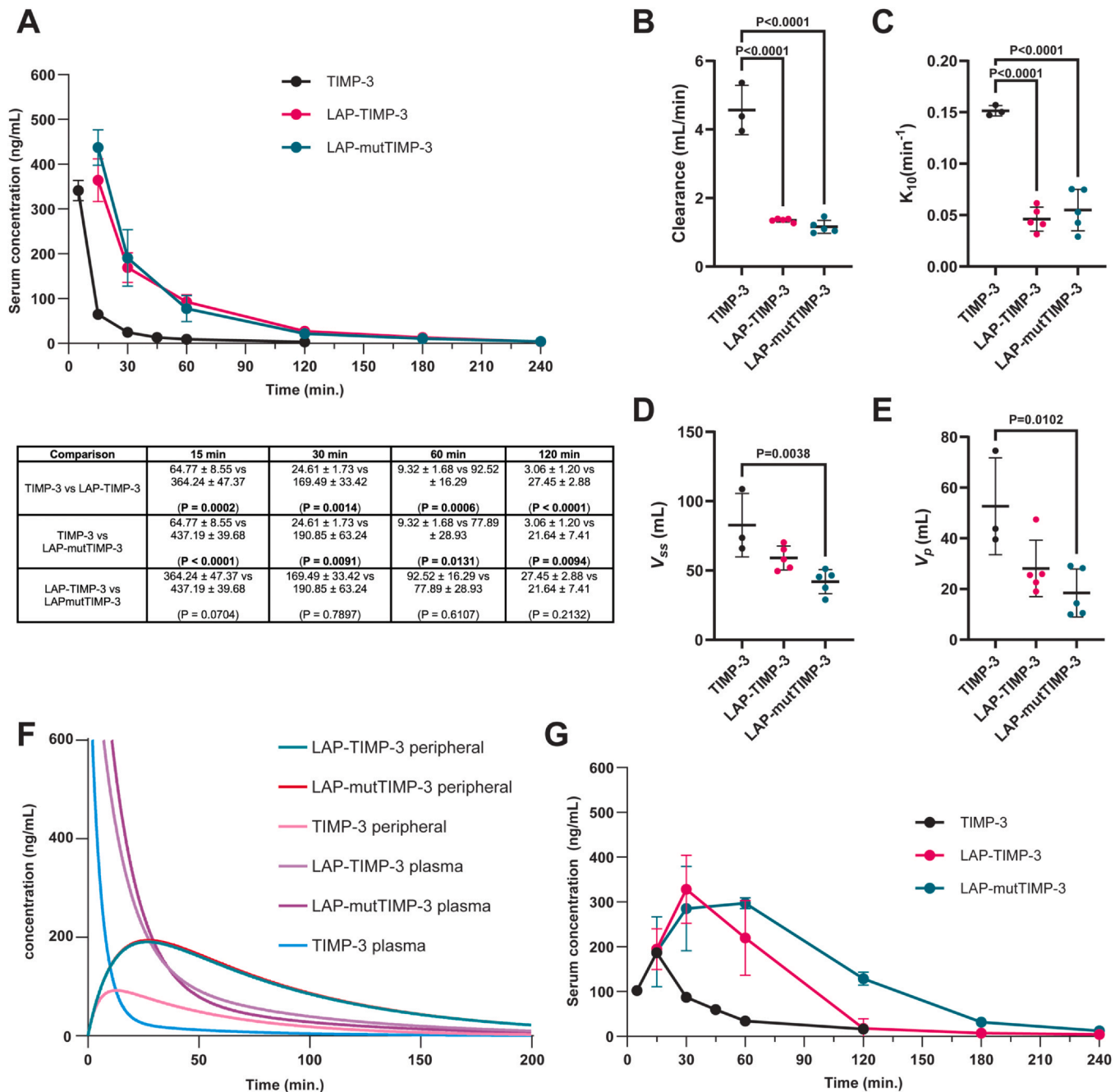
Systemic administration of LAP-TIMP-3/LAP-mutTIMP-3 reduced aggrecanase activity in the articular cartilage of knee joints in murine models of OA

Having established that the LAP-TIMP-3 protein entered the circulation and was present in knee joints up to at least 24 h after intraperitoneal injection, the effects of LAP-TIMP-3 proteins on aggrecanase activity in the articular cartilage of joints affected by OA were tested *in vivo*.

Previous studies have shown therapeutic potential of [-1A]TIMP-3 in both spontaneously-occurring [23] and surgical [38] mouse models of OA using transgenic mice. We tested our LAP proteins in similar models, using young (18 weeks) STR/Ort mice where OA develops spontaneously from about 15 weeks of age [39–41] and the DMM surgical model of OA [33]. In addition, the right legs of the STR/Ort mice were loaded to accelerate progression of focal lesions as an additional model of post-traumatic OA [32]. As levels of TIMP-3 required to achieve inhibition of aggrecanase in joints affected by OA were unknown, a 1 mg/kg dose was used given that, at this dose the proteins were still detectable in the joints 24 h post-administration (Fig. 3c). STR/Ort mice aged 18 weeks were treated thrice weekly for 3 weeks. A relatively low level of disease was present in the knees of these mice as expected at this age, with a high level of variability in disease score in both loaded (mean OARSI score: 10.5 ± 2.5) and unloaded (mean OARSI score: 9.8 ± 2) limbs. However, immunolabeling of the NVTEGE epitope of aggrecan showed lower levels of aggrecanase activity in the articular cartilage of most of the mice treated with the LAP-TIMP-3 proteins, in both unloaded and loaded limbs, compared with untreated control mice (Fig. 4a and b).

Two experimental protocols were used for the DMM model as described in Figure 1. Operated mouse knees collected 8 weeks after DMM surgery showed extensive signs of OA, with some knees reaching OARSI scores of > 25 and with extensive articular cartilage loss (Fig. 5a and Supplementary Fig. 4a). The mean OARSI score for the untreated DMM surgery group of mice was 16 ± 1.7 . Immunohistochemical analysis of aggrecanase activity in the articular cartilage of these limbs was not performed as this neoepitope is no longer present at this timepoint [38].

Surprisingly, analysis of the contralateral (un-operated) knees also showed low disease scores (mean OARSI score: 8.7 ± 1.04 ; Fig. 5b and Supplementary Fig. 4b) with joint deterioration present primarily on the lateral aspect of these knees (Supplementary Fig. 4c). This contrasted with the ipsilateral operated knees where OA occurred primarily on the medial aspect. Immunohistochemistry

**Fig. 2**

Effect of the LAP on the pharmacokinetics of TIMP-3. A. Purified TIMP-3 (*n*=3), LAP-TIMP-3 (*n*=5) or LAP-mutTIMP-3 (*n*=5) were injected intravenously at a concentration of 1 mg/kg into mice and blood samples collected at the times indicated. Concentrations of recombinant proteins in mouse sera were quantified by TIMP-3 ELISA or LAP ELISA. *P* values were determined using Tukey's post-hoc pairwise comparisons following a significant interaction identified by two-way ANOVA (*p* < 0.0001). (B–F) Pharmacokinetic analyses of LAP-TIMP-3 proteins using a 2-compartment model. B. Clearance: volume of plasma cleared of the recombinant proteins per unit time (Mean ± SD: TIMP-3 4.6 ± 0.72, LAP-TIMP-3 1.3 ± 0.05, LAP-mutTIMP-3 1.2 ± 0.19); C. K_{10} : elimination rate constant for each protein (Mean ± SD: TIMP-3 0.15 ± 0.005, Purified-TIMP-3 0.046 ± 0.01, LAP-mutTIMP-3 0.05 ± 0.02); D. V_{ss} : apparent volume of distribution for each protein at steady state (Mean ± SD: TIMP-3 82.7 ± 22.8, LAP-TIMP-3 59 ± 8.7, LAP-mutTIMP-3 41.9 ± 8.7); E. V_p : apparent peripheral volume of distribution for each protein (Mean ± SD: TIMP-3 52.6 ± 19.1, LAP-TIMP-3 28.1 ± 11.1, LAP-mutTIMP-3 18.4 ± 9.5). *P* values were determined by Tukey's post-hoc pairwise comparisons following one-way ANOVA (see Table 1 for details). F. Simulation of plasma and peripheral concentrations as a function of time. G. Purified TIMP-3 (*n*=1), LAP-TIMP-3 (*n*=5) or LAP-mutTIMP-3 (*n*=4) were injected intraperitoneally into mice at a concentration of 1 mg/kg and blood samples collected at the times indicated. Concentrations of recombinant proteins in mouse sera were quantified as described above.

Parameter	TIMP-3	LAP-TIMP-3	LAP-mutTIMP-3	LAP-TIMP-3 vs TIMP-3	LAP-mutTIMP-3 vs TIMP-3	LAP-TIMP-3 vs LAP-mutTIMP-3	One-way ANOVA Significance
Cl (mL/min)	4.568 ± 0.719	1.352 ± 0.053	1.160 ± 0.189	P < 0.0001	P < 0.0001	P=0.6641	P < 0.0001
V _c (mL)	30.09 ± 3.908	30.91 ± 8.106	23.48 ± 8.709				P=0.3160
V _{ss} (mL)	82.73 ± 22.85	59.01 ± 8.673	41.92 ± 8.774	P=0.0710	P=0.0038	P=0.1392	P=0.0049
V _p (mL)	52.64 ± 19.13	28.10 ± 11.14	18.43 ± 9.486	P=0.0565	P=0.0102	P=0.4731	P=0.0126
k ₁₀ (min ⁻¹)	0.151 ± 0.005	0.046 ± 0.011	0.054 ± 0.020	P < 0.0001	P < 0.0001	P=0.6316	P < 0.0001
k ₁₂ (min ⁻¹)	0.043 ± 0.004	0.023 ± 0.010	0.016 ± 0.011	P=0.0411	P=0.0098	P=0.5847	P=0.0115
k ₂₁ (min ⁻¹)	0.026 ± 0.004	0.024 ± 0.005	0.018 ± 0.004				P=0.0961
α (min ⁻¹)	0.202 ± 0.009	0.079 ± 0.020	0.076 ± 0.030	P < 0.0001	P < 0.0001	P=0.9803	P < 0.0001
β (min ⁻¹)	0.019 ± 0.002	0.014 ± 0.002	0.013 ± 0.003				P=0.0686
MRT _t (min)	17.90 ± 2.062	43.56 ± 5.490	37.21 ± 11.48	P=0.0038	P=0.0212	P=0.4582	P=0.0046
MRT _c (min)	6.605 ± 0.215	22.86 ± 5.963	20.68 ± 8.758	P=0.0193	P=0.0401	P=0.8663	P=0.0019
MRT _p (min)	11.30 ± 2.266	20.70 ± 7.746	16.53 ± 9.369				P=0.2933

Table 1

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Mean (± SD) pharmacokinetic parameters for TIMP-3, LAP-TIMP-3 and LAPmutTIMP-3 formulations. Cl, total body Clearance; V_c, Volume of Distribution of the central compartment; V_{ss}, Volume of Distribution at steady state; V_p, Volume of Distribution of the peripheral compartment; k₁₀, elimination rate constant; k₁₂ and k₂₁, transfer rate constants from the 1st to 2nd, and 2nd to 1st compartment respectively; α, distribution rate constant; β, post-distribution rate constant; MRT_t, total Mean Residence Time; MRT_c, Mean Residence Time in the central compartment; MRT_p, Mean Residence Time in the peripheral compartment. Statistical comparisons were performed using one-way ANOVA followed by Tukey's post-hoc test.

showed significantly less NVTEGE labelling, the neo-epitope of aggrecan generated by aggrecanase activity, in contralateral knees, with mean differences of 535 [95% CI: 336, 733] for LAP-TIMP-3 and 522 [95% CI: 323, 720] for LAP-mutTIMP-3 versus untreated control knees (Fig. 5c and d).

Operated knees of mice collected 2 weeks after DMM had a much milder OA severity than those collected at 8 weeks post-surgery (Supplementary Fig. 5a and 5b). Mice were treated with the TIMP-3 proteins for 1 week prior to DMM surgery, with additional treatment for 2 weeks post-surgery. Treatment with the LAP proteins resulted in lower mean levels of aggrecanase activity, but this did not reach statistical significance (Supplementary Fig. 5c).

An important consideration when using recombinant proteins *in vivo* is whether they induce an immune response, particularly with repeated administration. We found antibodies against the recombinant proteins (Supplementary Fig. 6a), most likely associated with the His tag present in the LAP-TIMP-3 proteins (Supplementary Fig. 6b and c). Only one positive correlation of antibody level with disease score was observed, in the LAP-mutTIMP-3 group at 2 weeks post DMM surgery (Supplementary Figs. 7 and 8). There were no significant changes in pro-inflammatory cytokine gene expression in the spleen or liver of treated mice, apart from a decrease in *Il1b* expression in the spleen of mice treated with LAP-mutTIMP-3, indicating a lack of systemic inflammation (Supplementary Table 2).

Relationship between reduction in aggrecanase activity and disease scores

To determine whether the aggrecanase inhibitory activities of the LAP-TIMP-3 proteins translated into protection against OA, we analysed OARSI joint scores. There were no differences in OA severity between the right (loaded) and left (unloaded) knees in STR/Ort mice, and treatment with either of the LAP-TIMP-3 proteins had no effect (Fig. 6a and b). We also analysed three datasets from the mice used for DMM experiments: the operated limb collected from mice 2 weeks post DMM (Fig. 6c), and both operated (Fig. 6d) and contralateral limbs from mice collected 8 weeks post DMM (Fig. 6e). There were no differences in OA scores between treated and

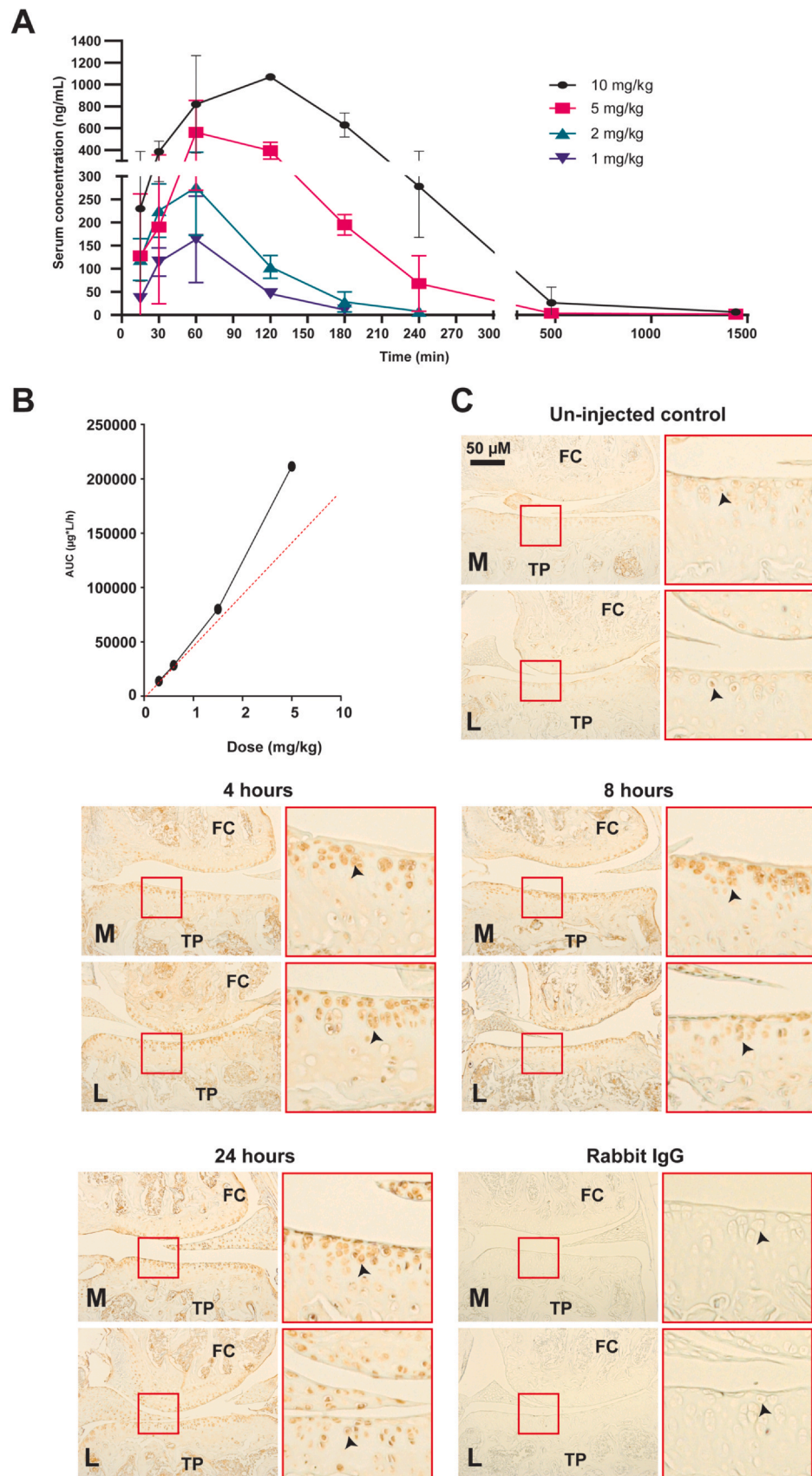
untreated mice in any of these datasets. Synovitis was assessed in the medial aspect of operated knees post DMM surgery. There were no differences in synovitis scores between treated and untreated mice at 2 weeks (Supplementary Fig. 9) or 8 weeks post DMM surgery (Supplementary Fig. 10). Operated knees collected 8 weeks post DMM surgery were also assessed for any effects of treatment with LAP-TIMP-3 proteins on the subchondral bone by micro-computed tomography (micro-CT) analyses. There were no changes detected in the density or architecture of the subchondral bone in response to surgery or treatment (Supplementary Fig. 11).

As OARSI scoring is not designed to discriminate disease severity in early-stage disease, we developed a more granular scoring scale (detailed in supplementary Table 1) for these samples. These analyses showed that although mean OA scores were lower in mice treated with LAP-TIMP-3 than in the untreated control group in operated knees collected 2 weeks post DMM or in contralateral knees collected 8 weeks post DMM, this did not reach statistical significance (Fig. 7). However, in both datasets, knees from mice treated with LAP-mutTIMP-3 had significantly reduced scores (2 weeks post DMM: mean difference 4.2 [95% CI: 0.5, 7.9]; contralateral limbs 8 weeks post DMM: mean difference 6.7 [95% CI: 1.6, 11.8] (Fig. 7).

Discussion

The efficacy of [-1A]TIMP-3 in reducing cartilage degradation in spontaneous OA [23] and in the DMM model of OA [38] has been demonstrated. However, these studies relied on transgenic mice overexpressing [-1A]TIMP-3, partly due to the difficulty of producing sufficient TIMP-3 for testing *in vivo*. Our prior synthesis of LAP-TIMP-3 protein [25] has a more translational approach to selectively inhibit aggrecanase activity, mimicking [-1A]TIMP-3. These novel data report the therapeutic potential of systemically administered recombinant TIMP-3 proteins for OA treatment.

We determined the pharmacokinetics of LAP-TIMP-3 *in vivo* for the first time. LAP technology enabled systemic protein administration via both intravenous and intraperitoneal routes with results showing either method could be used therapeutically. The main pharmacokinetic



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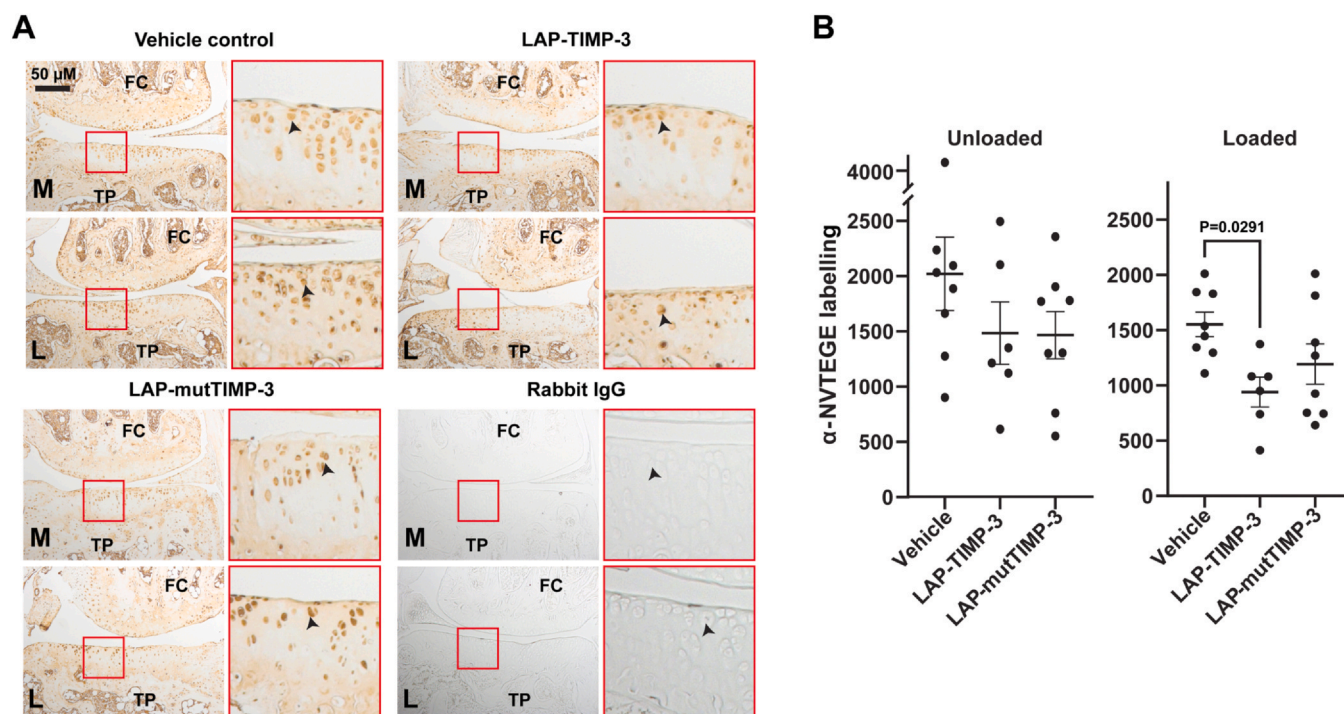
Fig. 3

Pharmacokinetics and tissue distribution of LAP-TIMP-3 No-CLV in mice. A. Varying concentrations of recombinant LAP-TIMP-3 No-CLV were administered to mice ($n=2$ per concentration of protein) intraperitoneally and blood samples collected from the tail vein at time intervals over 24 h. Levels of LAP-TIMP-3 No-CLV were measured in the serum of these mice via TIMP-3 or LAP ELISA. Data shown as mean $\pm$ SD. B. Steady state concentration curve generated from data shown in panel A. C. Sections from control (un-injected mice) or mice injected with 1 mg/kg of LAP-TIMP-3 No-CLV collected 4, 8 or 24 h after injection. Knees were fixed, sectioned and immunolabeled with anti-TIMP-3 antibody. L: lateral aspect of joint. M: medial aspect of joint. FC: femoral condyle. TP: tibial plateau. 1.5x magnified inset shows TIMP-3 staining in the pericellular matrix of the articular cartilage as indicated by the black arrows. Scale bar = 50 μ m.

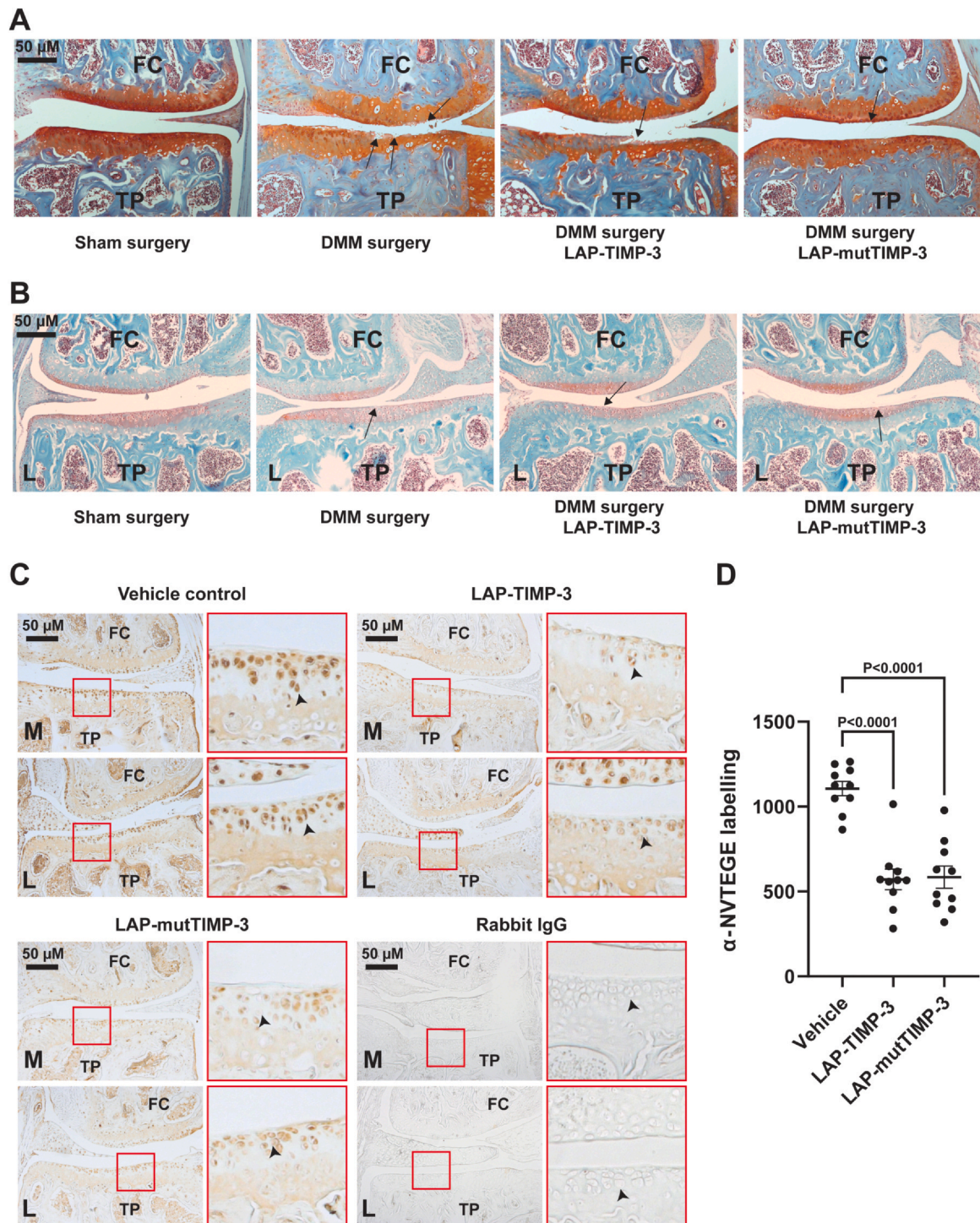
effect of LAP conjugation was decreased clearance and increased MRT of TIMP-3. This extended circulatory MRT, especially after intraperitoneal administration, prolongs the time LAP-TIMP-3 remains at elevated blood levels, increasing the chance of delivery to joint tissues.

Notably, LAP-TIMP-3 was found in the articular cartilage up to 24 h after intraperitoneal injection, showing it reaches the joint via the circulation and is retained within the cartilage for at least 24 h. Delivery of this large molecule across the joint capsule and synovial membrane was expected, as aggrecanase-specific antibodies also penetrate the knee joint cartilage following systemic administration in OA mice [42]. The proteins were not detected in non-joint tissues 24 h after injection, suggesting that they do not accumulate in these tissues.

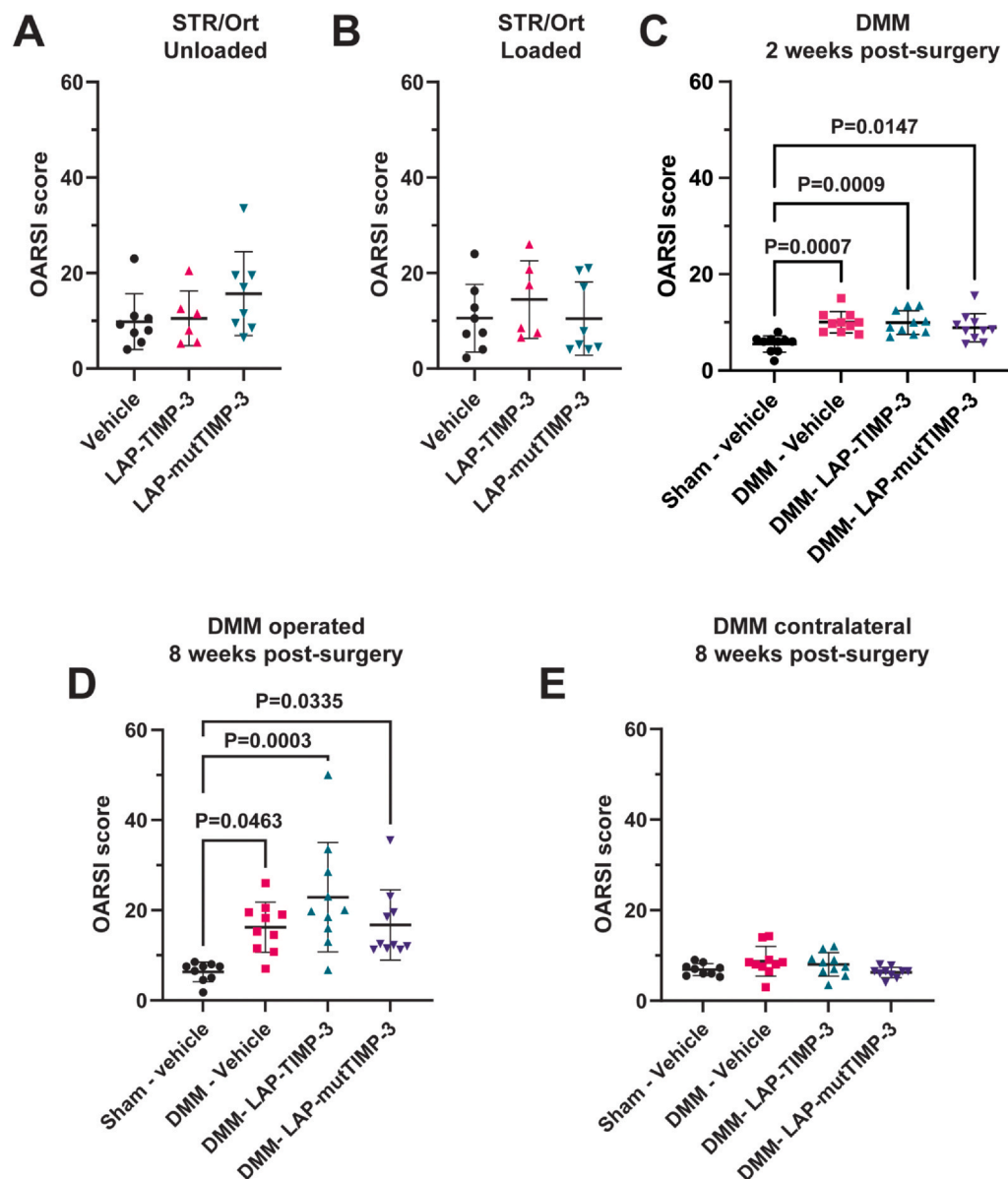
The LAP-TIMP-3 proteins are designed to inhibit aggrecanase activity, which occurs early in OA [43,44]. Sufficient inhibition may prevent collagen exposure to MMPs, reducing cartilage ECM degradation [45]. This aligns with reduced OA severity seen in mice expressing [-1A]TIMP-3, which shares the same specificity as TIMP-3 released from LAP [23,38]. We demonstrate that treatment with LAP-TIMP-3 proteins reduced aggrecanase activity in OA-affected knees, with LAP-mutTIMP-3 having a stronger effect compared with LAP-wild-type TIMP-3. This indicates sufficient MMP activity in the joints to cleave the LAP-TIMP-3 molecules and release active TIMP-3. The clearest effect was observed in knees with mild disease (i.e. two weeks post DMM surgery and in the contralateral knees 8 weeks post DMM surgery). As this first *in vivo* study used dosing based on

**Fig. 4**

NVTEGE-positive labelling in articular cartilage in left (unloaded) knees of STR/Ort mice treated with LAP-TIMP-3 proteins. A. Sections from mice injected with 30 μ L of vehicle control (PBS) or with 1 mg/kg of LAP-TIMP-3 or LAP-mutTIMP-3 protein in a total volume of 30 μ L were collected at 21 weeks of age. Knees were fixed, sectioned and probed with anti-NVTEGE antibody. Rabbit IgG was used as negative control. L: lateral aspect of joint. M: medial aspect of joint. FC: femoral condyle; TP: tibial plateau. Arrows within magnified inset shows anti-NVTEGE immunolabeling in the pericellular matrix of articular chondrocytes. Scale bar = 50 μ m. B. Quantification of anti-NVTEGE immunolabeling in left (unloaded) and right (loaded) knees of STR/Ort mice. Lines show mean $\pm$ SD: Unloaded - Vehicle 2023 $\pm$ 942, LAP-TIMP-3 1485 $\pm$ 691, LAP-mutTIMP-3 1468 $\pm$ 607. Loaded - Vehicle 1553 $\pm$ 314, LAP-TIMP-3 940 $\pm$ 330, LAP-mutTIMP-3 1193 $\pm$ 517. P values were determined by Tukey's post-hoc pairwise comparisons following one-way ANOVA ($P=0.0291$). $n=8$ for vehicle control mice, $n=6$ for LAP-TIMP-3 treated mice and $n=8$ for LAP-mutTIMP-3 treated mice.

**Fig. 5**

Effect of 1 mg/kg of LAP-TIMP-3 or LAP-mutTIMP-3 protein on mouse knees 8 weeks post DMM surgery. A. Operated and B. Contralateral (unoperated) knees were sectioned and stained with safranin O/fast green. FC: femoral condyle; TP: tibial plateau. Arrows indicate damage to the articular cartilage. OARSI disease scores of representative images in operated knees: Sham = 0, DMM surgery = 4, DMM surgery / LAP-TIMP-3 = 4, DMM surgery / LAP-mutTIMP-3 = 1. OARSI disease scores of representative images in contralateral knees: Sham = 0, DMM surgery = 2, DMM surgery / LAP-TIMP-3 = 1, DMM surgery / LAP-mutTIMP-3 = 2. Scale bar = 50 μ m. C. Immunolabeling of contralateral knees from mice following DMM surgery on the ipsilateral side. Knees were fixed, sectioned and immunolabeled with anti-NVTEGE antibody. L: lateral aspect of joint. M: medial aspect of joint. FC: femoral condyle; TP: tibial plateau. Arrows within 1.5x magnified inset shows anti-NVTEGE immunolabeling in the pericellular matrix of articular chondrocytes. D. Quantification of anti-NVTEGE immunolabeling in the contralateral knees from mice following DMM surgery on the ipsilateral side. Lines show mean $\pm$ SD: Vehicle 1106 $\pm$ 132, LAP-TIMP-3 571 $\pm$ 191, LAP-mutTIMP-3 584 $\pm$ 206. $n=10$ for each group. P values were determined by Tukey's post-hoc pairwise comparisons following one-way ANOVA ($P < 0.001$).

**Fig. 6**

Osteoarthritis and Cartilage

Effect of treatment with LAP-TIMP-3 proteins on OARS disease scores in mouse knees. Knees were sectioned, stained with safranin O/fast green and scored using the OARS scoring scale. A. OARS scores in (left) unloaded knees of STR/Ort mice and B. (right) loaded knees of STR/Ort mice. Lines show mean $\pm$ SD: Unloaded - Vehicle 9.8 ± 5.8 , LAP-TIMP-3 10.5 ± 5.7 , LAP-mutTIMP-3 15.7 ± 8.7 ; Loaded - Vehicle 10.5 ± 7 , LAP-TIMP-3 14.5 ± 8.1 , LAP-mutTIMP-3 10.5 ± 7.6 . $n=8$ for vehicle control mice, $n=6$ for LAP-TIMP-3 treated mice and $n=8$ for LAP-mutTIMP-3 treated mice. Data were analysed by Kruskal-Wallis ($P=0.264$ for unloaded limbs and $P=0.447$ for loaded limbs). C. OARS scores in operated mouse knees 2 weeks post DMM surgery ($n=10$ per group). Lines show mean $\pm$ SD: Sham Vehicle 5.5 ± 1.7 , DMM Vehicle 10.05 ± 2.2 , LAP-TIMP-3 9.98 ± 2.4 , LAP-mutTIMP-3 8.9 ± 2.9 ; P-values as indicated were determined by Tukey's post-hoc pairwise comparisons following one-way ANOVA ($P=0.0003$). D. OARS scores in operated C57BL/6 mouse knees 8 weeks post DMM surgery. Lines show mean $\pm$ SD: Sham Vehicle 6.3 ± 2.2 , DMM Vehicle 16.2 ± 5.5 , LAP-TIMP-3 22.9 ± 12 , LAP-mutTIMP-3 16.7 ± 7.8 ; P-values as indicated were determined by Tukey's post-hoc pairwise comparisons following one-way ANOVA ($P=0.0008$). E. OARS scores in contralateral (un-operated) mouse knees 8 weeks post ipsilateral DMM surgery ($n=9$ for Sham-Vehicle; $n=10$ for DMM groups). Lines show mean $\pm$ SD: Sham Vehicle 6.8 ± 1.3 , DMM Vehicle 8.7 ± 3.3 , LAP-TIMP-3 8 ± 2.6 , LAP-mutTIMP-3 6.2 ± 1.2 . Symbols represent individual mice. Data were analysed by one-way ANOVA ($P=0.094$).

in vitro data, further optimisation is needed, but the effects on mild disease suggest clear therapeutic potential.

The protein dose used was informed by the detection of the proteins in cartilage 24 h after a 1 mg/kg dose. Although aggrecanase

activity decreased (as shown by reduced neo-epitope labelling), this did not always correspond to lower OA scores, especially in severe OA. Higher doses may be required to induce a greater therapeutic effect, as emphasised by previous studies using transgenic

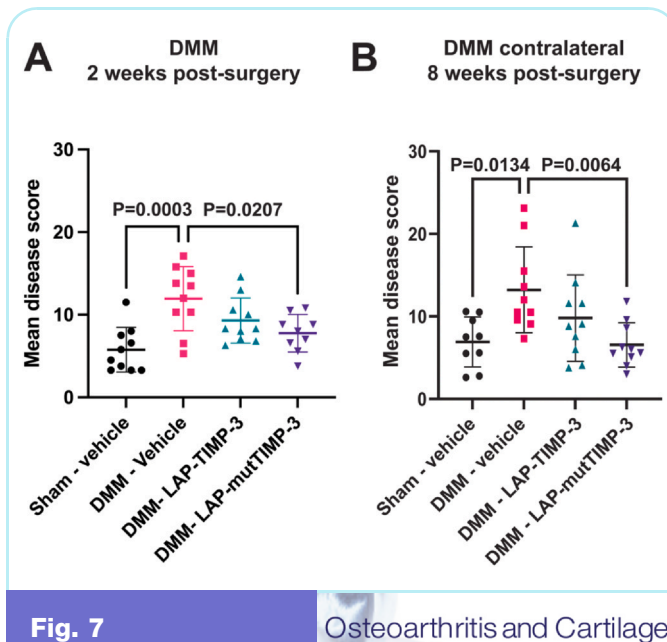


Fig. 7

Osteoarthritis and Cartilage

Effect of LAP-TIMP-3 proteins on OA disease scores in the knees of C57BL/6 mice. Disease was scored using a scoring scale designed for mild OA (described in the [supplementary methods](#)). Disease scores in A. operated knees 2 weeks post DMM surgery (n=10 per group, except for the LAP-mutTIMP-3 treated group where n=9 due to exclusion of 1 outlier as identified by the ROUT method). Lines show mean $\pm$ SD: Sham Vehicle 5.7 ± 2.7 , DMM Vehicle 12 ± 3.9 , LAP-TIMP-3 9.3 ± 2.7 , LAP-mutTIMP-3 7.8 ± 2.3 and B. knees contralateral to operated knees collected 8 weeks post DMM surgery (n=9 for sham-vehicle; n=10 for DMM groups). Lines show mean $\pm$ SD: Sham Vehicle 6.9 ± 3 , DMM Vehicle 13.2 ± 5.2 , LAP-TIMP-3 9.8 ± 5.2 , LAP-mutTIMP-3 6.5 ± 2.7 . Symbols represent individual mice. P-values as indicated were determined by Tukey's post-hoc pairwise comparisons following one-way ANOVA (P=0.0005) for A and (P=0.0043) for B.

[-1A]TIMP-3, where high expression was needed [23,38]. It is nonetheless highly promising that statistically significant protection against joint deterioration was evident in both operated knees 2 weeks post DMM surgery and contralateral knees of LAP-mutTIMP-3 treated mice subjected to ipsilateral DMM surgery.

Mild joint changes in contralateral knees provides insight into the effects of treatment at the early stages of joint dysfunction in relatively intact joints. Although OA was detectable in contralateral knees by OARSI scoring, a more accurate analysis of these very small changes in the articular cartilage was achieved using a more granular cartilage-focused scale. A further interesting phenomenon observed was the higher lateral compartment scores of the contralateral knees, whereas the ipsilateral knees had higher scores in the medial compartments. These differences could be caused by changes in gait, a phenomenon described in the DMM model [46] and in our non-invasive loading model [47]. Even mild changes in gait could cause these changes as the joint tissue appears to be extremely mechanosensitive, with DMM-induced up-regulation of known pathogenic genes abrogated by joint immobilisation [48].

The limitations of this study include the dosing regimen (injection 3 times per week for 3–6 weeks), which induced antibodies against the recombinant proteins. This was not wholly unexpected given the repeated administration but was not associated with systemic immune activation, as indicated by absent inflammatory cytokine

expression in the liver or spleen and no consistent correlation between antibody levels and OA severity. A further limitation is the use of male mice, which may reduce applicability to female patients who are disproportionately affected by OA. However, female mice show less joint degeneration in the DMM model [30,31,49] and for this first *in vivo* study, where optimal dose and timing of treatment was unknown, consistency within treatment groups was prioritised. Further studies will include both males and females to ensure translational relevance.

Furthermore, the LAP-mutTIMP-3 protein has significantly greater potential as a therapeutic agent than the LAP molecule containing the wild-type TIMP-3. Further work should optimise dose and timing, with higher concentrations likely required for meaningful effects.

CRediT authorship contribution statement

CRediT: Conceptualization: FS, GS, LT, BP, AP, LM; Data curation: FS, LM; Formal Analysis: FS, GS, MM, SM, AP, LM; Funding acquisition: LT, AP, LM; Investigation: FS, MM, JK, BP, LM; Methodology: FS, GS, MM, SM, BP, AP, LM; Project administration: FS, LT, AP, LM; Resources: KAS; Software: SM; Supervision: AP, LM; Writing – original draft: LM; Writing – review & editing: FS, GS, MM, SM, JK, KAS, LT, BP, AP, LM.

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Competing interests

The authors declare that they have no competing interests.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.joca.2025.09.002](https://doi.org/10.1016/j.joca.2025.09.002).

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