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R399E, A Mutated Form of Growth and Differentiation Factor 5, for Disease Modification of Osteoarthritis

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Objective. To preclinically characterize a mutant form of growth and differentiation factor 5, R399E, with reduced osteogenic properties as a potential disease-modifying osteoarthritis (OA) drug.

Methods. Cartilage, synovium, and meniscus samples from patients with OA were used to evaluate anabolic and antiinflammatory properties of R399E. In the rabbit joint instability model, 65 rabbits underwent transection of the anterior cruciate ligament plus partial meniscectomy. Three intraarticular (IA) R399E doses were administered biweekly 6 times, and static incapacitance was determined to assess joint pain. OA was evaluated 13 weeks after surgery. In sheep, medial meniscus transection was performed to induce OA, dynamic weight bearing was measured in-life, and OA was assessed after 13 weeks.

Results. Intermittent exposure to R399E (1 week per month) was sufficient to induce cell proliferation and release of anabolic markers in 3-dimensional chondrocyte cultures. R399E also inhibited the release of interleukin-1β (IL-1β), IL-6, and prostaglandin E_2 from cartilage with synovium, meniscal cell, and synoviocyte cultures. In rabbits, the mean difference (95% confidence interval [95% CI]) in weight bearing for R399E compared to vehicle was –5.8 (95% confidence interval [95% CI] –9.54, –2.15), –7.2 (95% CI –10.93, –3.54), and –7.7 (95% CI –11.49, –3.84) for the 0.6, 6, and 60 µg doses, respectively, 6 hours after the first IA injection, and was statistically significant through the entire study for all doses. Cartilage surface structure improved with the 6-µg dose. Structural and symptomatic improvement with the same dose was confirmed in the sheep model of OA.

Conclusion. R399E influences several pathologic processes contributing to OA, highlighting its potential as a disease-modifying therapy.

INTRODUCTION

Osteoarthritis (OA) is the most common painful and progressive joint disorder, with currently more than 300 million cases of knee and hip OA worldwide (1). The disease pathology progresses in all tissues of the affected joint and is characterized by cartilage and meniscus degradation, deformation of ligaments, and subchondral bone changes, resulting in severe pain that significantly affects a patient's mobility, sleep, and ability to work. The pathologic mechanisms that result in the vicious cycle of progressive OA are thought to arise from an imbalance between anabolic and catabolic processes (2–4). While several therapeutic concepts have been investigated for their disease modifying potential (5), the American College of Rheumatology and the Arthritis Foundation recently concluded that the management of OA remains limited to exercise and topical or oral nonsteroidal antiinflammatory drugs (NSAIDs) (6). Currently, there is no treatment available for OA with benefits for pain and joint structure, and thus it remains a substantial unmet medical need.

Growth and differentiation factor 5 (GDF5), a member of the transforming growth factor β family, is known to be important for cartilage formation during skeletal development (7) and several reports indicate that supplementation with GDF5 could be beneficial for patients with OA. First, using adult primary chondrocytes in vitro, it was demonstrated that GDF5 increases matrix production (8,9) and decreases expression of matrix metalloproteinase 13

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(MMP13) and ADAMTS4, enzymes that degrade cartilage matrix (10). Moreover, a GDF5 polymorphism leading to reduced expression of GDF5 was associated with OA susceptibility (11–13) and specifically with knee pain (14). In addition, the therapeutic potential of intraarticular (IA)–administered GDF5 has been investigated in vivo in a rat medial meniscus transection model of OA, demonstrating that GDF5 significantly preserved cartilage structure but also substantially enhanced osteophyte growth (15). Similarly, in vitro, GDF5 enhanced both chondrogenesis and hypertrophy of mesenchymal stem cells (16,17).

To reduce the osteogenic properties of GDF5, 18 mutants with mutations in residues located in the bone morphogenetic protein receptor 1A (BMPR1A) and BMPR1B binding site of GDF5 were generated via site-directed mutagenesis and profiled in cell culture and biochemical assays (18). The mutant R399E, in which the residue R at the position 399 was substituted with an E in sequence AAH32495, showed similar chondrogenic properties to GDF5 (19) but lower hypertrophic and osteogenic properties (20).

In this preclinical study, we evaluate the therapeutic potential of R399E for OA. We first used human OA chondrocytes in 3-dimensional (3-D) cultures to demonstrate that R399E induces cell proliferation and the release of anabolic markers. We also show that R399E inhibits release of the inflammatory mediators interleukin-1β (IL-1β), IL-6, and prostaglandin E_2 (PGE_2) from different tissues of patients with OA including cartilage, synovium, and meniscus. Finally, in a rabbit model of surgically induced OA, IA administration of R399E elicited symptom relief within 6 hours of the first injection, which was maintained over the entire 13-week study. In the same study, structural improvement was shown by improved gross morphology with the same dose in the same animals. Structural and symptomatic improvements were confirmed in a sheep model of OA.

MATERIALS AND METHODS

Human subjects. Articular cartilage, synovium, and meniscus tissue samples were obtained from 15 individual donors who underwent total knee replacement at the Elisabethenstift, Darmstadt, Germany. All samples were anonymized, and all patients provided written informed consent prior to surgery. Use of the tissue samples was approved by the Landesärtzekammer Hessen, Germany (FF24/2015). All documents are stored at the Elisabethenstift Hospital.

Human OA chondrocyte culture. Human OA knee chondrocytes were obtained from 4 donors and encapsulated in alginate, as previously described by Mang et al (19). Briefly, cartilage was digested with collagenase (collagenase NBG4; Serva), and the resulting cell suspension was filtered, washed, and cultured for 7 days in monolayer in high-glucose Dulbecco's modified Eagle's medium (DMEM) (Gibco), 10% fetal bovine serum (Biochrom), 50 μg/ml ascorbate 2-phosphate (Sigma), and 400 μ*M* proline (Sigma). Cells were then encapsulated in alginate and 5–7 alginate beads (corresponding to 1–2 million cells) were transferred to a 24-well plate in 1 ml of culture medium adjusted at 380 mOsm and supplemented with R399E (100, 300, or 1,000 ng/ml) or 6.25–12.5 μ*M* HCl (control). After 4 weeks of culture, the alginate beads were dissolved, and the cell concentration was evaluated with a Vicell XR analyzer (Beckman Coulter; n = 2–4 per donor). Glycosaminoglycan (GAG; a component of proteoglycan) (n = 6–8 culture replicates per donor) concentration in the dissolved alginate supernatant was measured with the 1,9-dimethylmethylene blue assay (21). Levels of propeptides from type II collagen (PRO-C2) and type VI collagen (PRO-C6), which reflect collagen production (n = 3 per donor), were measured in the medium at the end of culture, according to methods previously described by Luo et al (22) and Sun et al (23), respectively. Data were normalized by dividing all values by the averaged values of the respective control.

Human cartilage with synovium coculture. Human knee cartilage explants (4-mm diameter) with synovial membrane (20–25 mg) from 2 donors were freshly prepared. The cartilage and synovium were cocultured in a 48-well plate in 300 μl Ham's F12 medium (PAN Biotech) with ascorbate 2-phosphate, amphotericin B (PAN Biotech) and penicillin/streptomycin (Invitrogen). The cocultures were left untreated or were treated with 100 or 300 ng/ml R399E for 4 days. As controls, cartilage and synovial membrane explants were cultured alone. The number of technical replicates for all groups was 4. IL-1β, IL-6, tumor necrosis factor (TNF), and IL-8 were measured in the medium on day 4 with the human proinflammatory panel II, 4-plex on a Meso Scale Discovery platform (kit K15054D), and PGE_2 was measured using the PGE_2 kit from Cisbio.

Human meniscus cell culture. Primary human meniscal cells were prepared as previously described by Verdonk et al (24). Briefly, the menisci were digested with 0.4% collagenase at 37°C for 16 hours. The resultant cell suspension was filtered and washed, and the cells were incubated in a 96-well plate at 10,000 cells/well with Ham's F12 containing 1% penicillin/streptomycin, 1% amphotericin, and 10% fetal calf serum. Cells were first cultured for 1 week and were stimulated with 10 ng/ml recombinant human nerve growth factor (NGF; Merck Millipore) and were treated with 0.1 μ*M* dexamethasone (Sigma-Aldrich), 0.1 μ*M* triamcinolone (Chemcruz), 375 ng/ml anti-NGF antibody, 300 ng/ml R399E, or were left untreated (negative control). After 2 or 7 days, PGE_2 was measured in the medium.

Pharmacokinetics in rabbits. NZW rabbits (n = 39), 4–5 months of age, were used. Whole blood was collected from 3 rabbits per group per time point at the following time points: 1, 3, 7, 24, 48, 72, 120, 168, 240, 336, 408, 504, and 672 hours after

a single IA injection of 2 μg R399E per joint. Synovial fluid lavage was performed directly after euthanasia. Afterward, the injected right stifle joint was collected intact and flash frozen. Pharmacokinetic (PK) parameters were determined for each matrix. More details are provided in Supplementary Methods, available on the *Arthritis & Rheumatology* website at https://onlinelibrary.wiley.com/doi/10.1002/art.42343.

Surgically induced OA in rabbits. Six-month-old female NZW rabbits (Crl:KBL; Charles River Laboratories) underwent transection of the anterior cruciate ligament and partial resection of the medial meniscus in the right knee. In 2 pharmacology studies, rabbits (n = 65 in OA study 1; n = 63 in OA study 2) received IA injections in the operated joint with 0.6 μg (n = 16 and 12, respectively), 6 μg (n = 16 and 13), or 60 μg (n = 16 and 13) R399E or with vehicle (n = 17 and 13) administered every 2 weeks, starting 1 week postsurgery. In the second OA study, a fifth group (n = 12) was injected IA with triamcinolone (Zentiva) at week 1 and intravenously with anti-NGF antibody at weeks 5 and 7. Static incapacitance was measured contact-free to ensure observer independence with a custom-made device prior to surgery (Supplementary Methods, https://onlinelibrary.wiley.com/doi/10.1002/art.42343), 1 week after surgery but before the first injection, 6 hours after the first injection, then every 2 weeks preinjection, and once after the last injection. After 13 weeks, animals were euthanized, and cartilage was evaluated by gross morphology. For the vehicle-treated and 6 μg R399E–treated groups, cartilage thickness and volume were evaluated by micro–computed tomography (micro-CT) using negative contrast. Additional details are provided in the Supplementary Methods. This study was performed in accordance with the German Animal Welfare Act and was approved by the regional authority of Darmstadt, Hesse, Germany.

Surgically induced OA in sheep. Adult female Welsh mountain sheep (n = 35; mean ± SD weight 46.9 ± 2.6 kg) were obtained from a licensed supplier (Newell Farms, Norfolk, UK), assigned blindly to each experimental group (n = 7) with blinding maintained throughout the experiment. To exclude husbandry bias, all sheep were housed together. Animals underwent a medial meniscus transection of the right knee. One week after surgery, animals received vehicle or 12 μg, 120 μg, or 1200 μg R399E administered IA every 4 weeks in the operated knee joints, and they were euthanized 13 weeks after surgery. Dynamic incapacitance was measured with an "Accugait" force plate (AMTI) prior to surgery and at preinjection at weeks 1, 2, 3, 5, and 9 after surgery. To quantify structural effects, the Kellgren/Lawrence (K/L) score (using radiographs), a gross morphology OA score (International Cartilage Repair Society score), and a modified Mankin score (histology analysis) were evaluated. Additional details including radiographic assessment scoring details are provided in the Supplementary Methods (https://onlinelibrary.wiley.com/doi/10.1002/art.42343). This research was regulated under the Animals (Scientific Procedures) Act 1986 Amendment Regulations 2012 following ethical review by the University of Cambridge Animal Welfare and Ethical Review Body.

Statistical analysis. Statistical tests used were performed with GraphPad Prism version 7.00. The tests used are indicated in the figure legends.

RESULTS

Crystal structure of R399E. We solved the crystal structure of R399E in its homodimeric form (Supplementary Figure 1, https://onlinelibrary.wiley.com/doi/10.1002/art.42343). Both monomers together formed the content of the crystal asymmetric unit and were covalently bound by a disulfide bond between cysteine A465 (monomer A) and cysteine B465 (monomer B). Furthermore, the crystal structure revealed a salt bridge between Glu 399 and Arg 438 in both monomers.

Anabolic profile of R399E. Permanent exposure of 3-D cultures of human OA chondrocytes to R399E increased the number of cells which was significant at the highest concentration (Figure 1A). There was a concentration-dependent increase in GAG content in the 3-D constructs, which for all donors combined was significant at the 300 and 1,000 ng/ml doses (Figure 1B). R399E significantly induced the release of PRO-C2 and PRO-C6, markers for type II and VI collagen production, respectively (22,23) (Figures 1C and D). The responsiveness of individual donors was heterogeneous: donor 3 was most sensitive and responded significantly to R399E on all parameters, even at the lowest concentration of 100 ng/ml, whereas donor 1 exhibited only significant accumulation of GAG (Figure 1).

To determine whether permanent exposure of R399E is necessary to result in anabolic effects, we cultured chondrocytes in 3-D from a fifth donor for 2 months and treated them with R399E (300 ng/ml) either intermittently (1–3 weeks/month) or permanently (Supplementary Figure 2A, https://onlinelibrary.wiley.com/doi/10.1002/art.42343). Permanent exposure for 8 weeks, consistent with results from the 4-week study, resulted in a statistically significant increase in GAG and PRO-C2 content, and a significant increase in the number of chondrocytes. Intermittent exposure was also effective: levels of GAG, hydroxyproline (OHPro; a component of collagen), and PRO-C2 increased significantly compared to controls (Supplementary Figure 2A).

We further corroborated the effectiveness of intermittent exposure with 3-D porcine chondrocyte cultures with 20-week duration in which cells were exposed to R399E only during weeks 1, 5, and 9, resulting in significant increases in GAG and OHpro, and in the weight of the constructs at the end of week 20; constant exposure to R399E was more effective (Supplementary Figure 2B, https://onlinelibrary.wiley.com/doi/10.1002/art.42343).

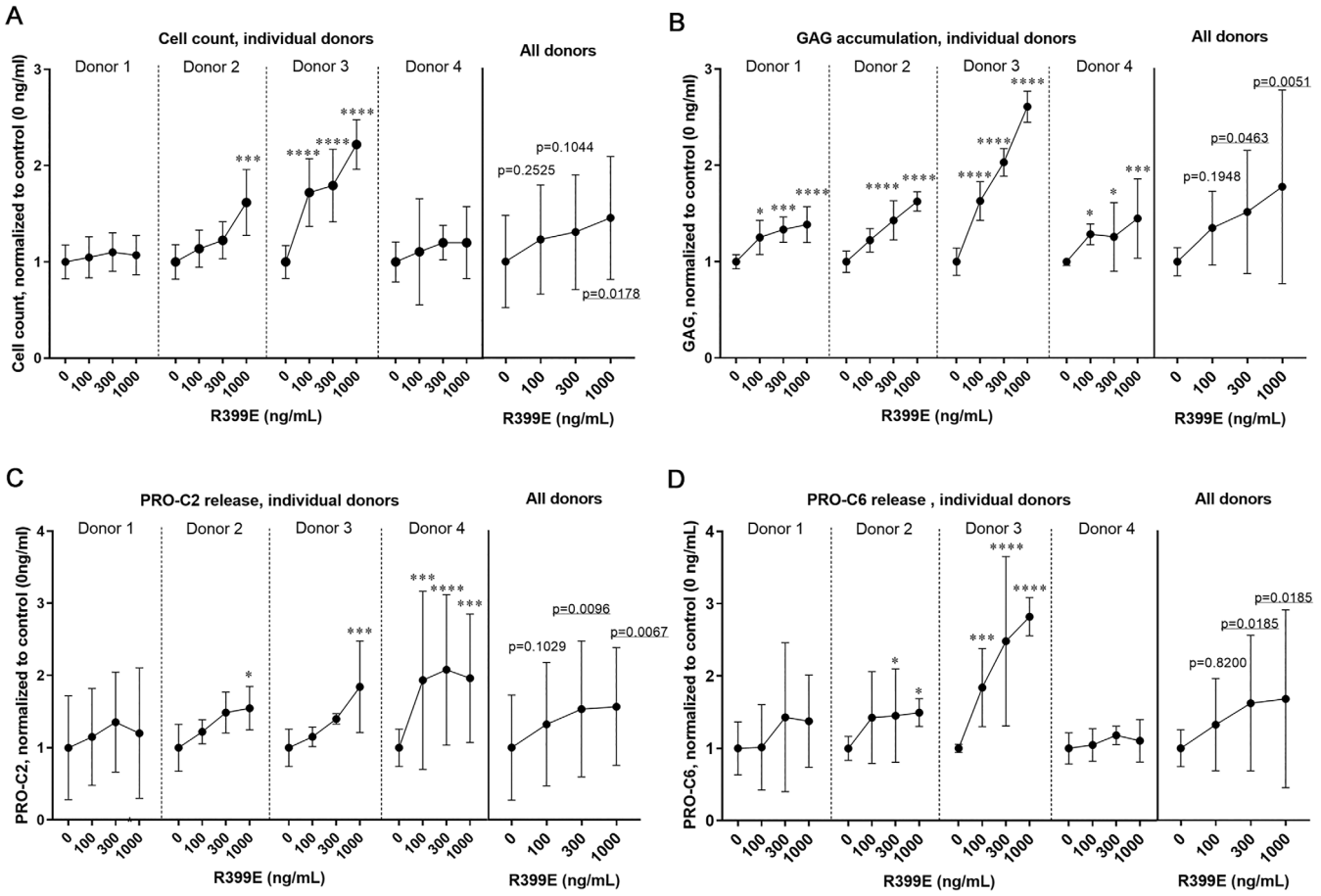

Figure 1. R399E increases number of chondrocytes, content of glycosaminoglycan (GAG), and release of collagen formation markers PRO-C2 and PRO-C6 in 3-dimensional cultures of human osteoarthritis (OA) chondrocytes. Human OA chondrocytes from 4 donors were cultured 4 weeks in alginate with 100, 300, or 1,000 ng/ml R399E or in the absence of R399E (medium with 12.5 μ*M* HCl). The cell number in the alginate beads (**A**), GAG (**B**), PRO-C2 (**C**), and PRO-C6 (**D**) release in the medium were evaluated. The results of the 4 donors are shown separately (n = 3–6 replicates/donor) or all together. Data were normalized to respective controls (see Supplementary Methods, https://onlinelibrary.wiley.com/doi/10.1002/art.42343), and bars show the mean and 95% confidence intervals. The treated groups were compared to the control group (0 ng/ml R399E) with two-way analysis of variance (ANOVA; for individual donors, with the variables being the donor and the concentration), with matched data ANOVA followed by Dunnett's test to correct for multiple comparisons (for all donors together for **A**–**C**), or nonparametric Friedmann test followed by a Dunn's test for PRO-C6. * = $P < 0.05$; *** = $P < 0.001$; **** = $P < 0.0001$, for individual donors. For all donors together, *P* values are indicated on the graphs and are underlined when <0.05.

Histologic evaluation of the constructs confirmed these findings (Supplementary Figure 2B).

Hypertrophic and osteogenic capacity of R399E. In human OA chondrocytes, we observed that R399E did not significantly increase type X collagen expression but did significantly increase type II collagen expression (Supplementary Figure 3, https://onlinelibrary.wiley.com/doi/10.1002/art.42343). This result indicates that R399E has no important hypertrophic potential in human OA chondrocytes.

In addition, we investigated whether R399E induces ectopic bone formation in a dedicated in vivo study in rabbits. Rabbits (n = 12) received injections of vehicle or of 6 μg or 60 μg R399E (n = 4/treatment group). Animals were injected intramuscularly in the left and right lumbar muscle and periarticularly subcutaneously on the right and left leg on days 0, 14, and 29; animals survived through day 42 (Supplementary Methods, https://onlinelibrary.wiley.com/doi/10.1002/art.42343). Injections of R399E resulted in no histologic or radiographic evidence of soft tissue mineralization/calcification or ectopic bone formation, regardless of dose.

Effects of R399E on OA-related inflammatory processes. Culture of cartilage explants together with synovial membrane from the same patients led to a significant increase in IL-1β in the supernatant (Figure 2A). This increase was significantly inhibited by R399E, with 100 ng/ml and 300 ng/ml R399E in donor 1 and with 300 ng/ml in donor 2 (Figure 2A). Likewise, PGE_2 release was found to be significantly enhanced in the cartilage–synovium cocultures; R399E significantly reduced the production of PGE_2 in donor 1 but not in donor 2 (Figure 2B).

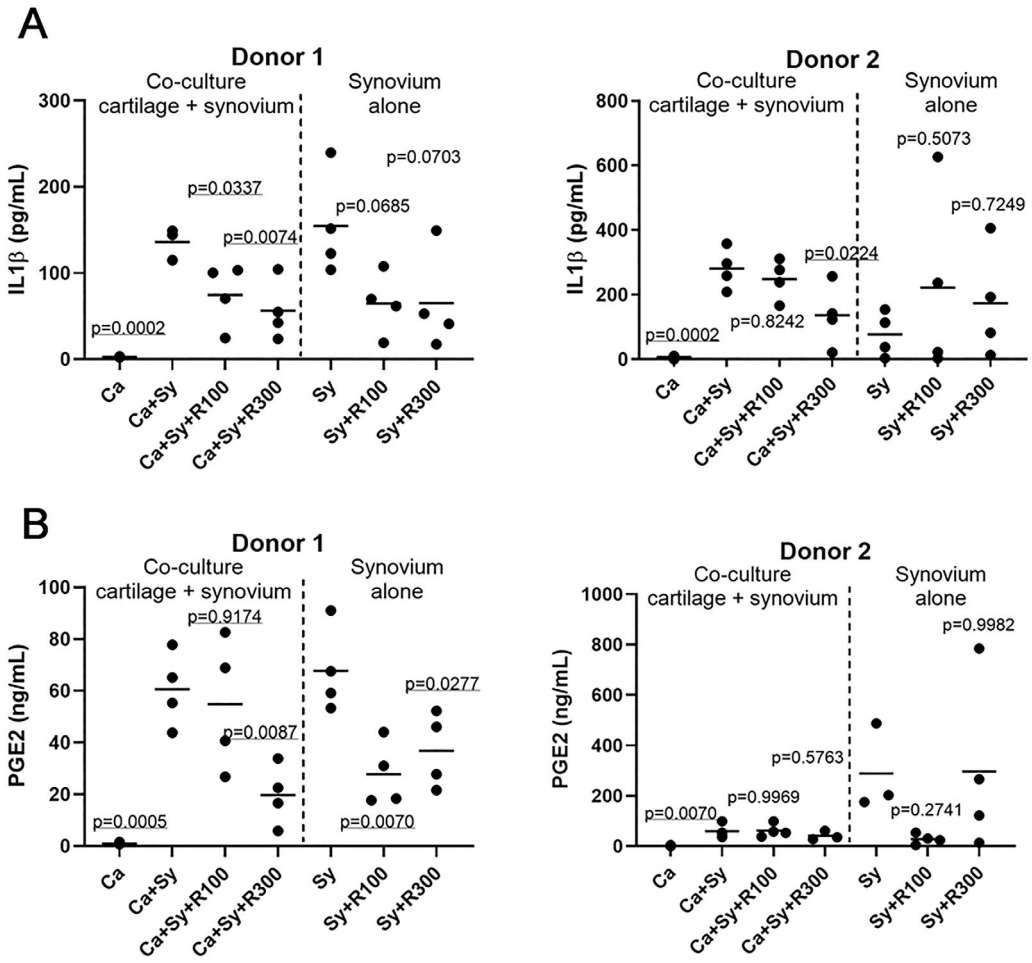

Figure 2. R399E reduces release of inflammatory mediators interleukin-1β (IL-1β) and prostaglandin E_2 (PGE_2) and of GAG from human cartilage–synovium coculture. Cartilage (Ca) and synovium (Sy) explants from the same donors were cultured alone or together (Ca + Sy) in the absence (culture medium with no added compound) or presence of R399E at 100 or 300 ng/ml (R100 and R300, respectively). IL-1β (**A**) and PGE_2 (**B**) were evaluated in the medium on day 4. Two donors were used (n = 4 data points/donor). Bars show the mean. A one-way ANOVA followed by Dunnett's test to correct for multiple comparisons was used to compare the Ca + Sy group to the 3 other groups for the coculture, and the Sy group to the 2 other groups for the culture of synovium alone. *P* values are underlined when $P < 0.05$. See Figure 1 for other definitions.

When culturing synovial membrane alone, we found a substantial spontaneous release of IL-1β and PGE_2, which could both be significantly attenuated by R399E in donor 1 but not in donor 2 (Figure 2). IL-6, TNF, and IL-8 were not modulated, i.e., their levels did not increase significantly when synovium was added to cartilage cultures.

We further evaluated the inflammatory potential of R399E in various cell types. We found that the addition of NGF to the culture of isolated cells from primary human OA menisci induced the release of PGE_2 and that this PGE_2 induction was inhibited by R399E, with an effect size comparable to that of the steroids dexamethasone or triamcinolone (Supplementary Figure 4A and B, https://onlinelibrary.wiley.com/doi/10.1002/art.42343).

Antiinflammatory effects of R399E were corroborated in the synoviocyte cell line SW982 and in isolated primary human OA synoviocytes (Supplementary Methods, https://onlinelibrary.wiley.com/doi/10.1002/art.42343). In both assays, R399E significantly reduced IL-6 and IL-1β release; the lower concentration of 300 ng/ml was more effective in SW982 cells, whereas 900 ng/ml was more effective in primary synoviocytes (Supplementary Figure 5A and B, https://onlinelibrary.wiley.com/doi/10.1002/art.42343).

Exposure levels of R399E after IA injection in rabbits. To evaluate the exposure levels of R399E and in support of further understanding the PK profile in our preclinical models, R399E was administered via IA injection (2 μg) in rabbits. R399E exposure decreased rapidly in synovial fluid during the first 24 hours, with concentrations measured 7 hours postinjection at only 15% of the concentration measured after 1 hour (Figure 3A). For a detailed description of assessment of PK of R399E, see the Supplementary Methods (https://onlinelibrary.wiley.com/doi/10.1002/art.42343). However, the decline in R399E levels slowed, and R399E remained detectable in synovial

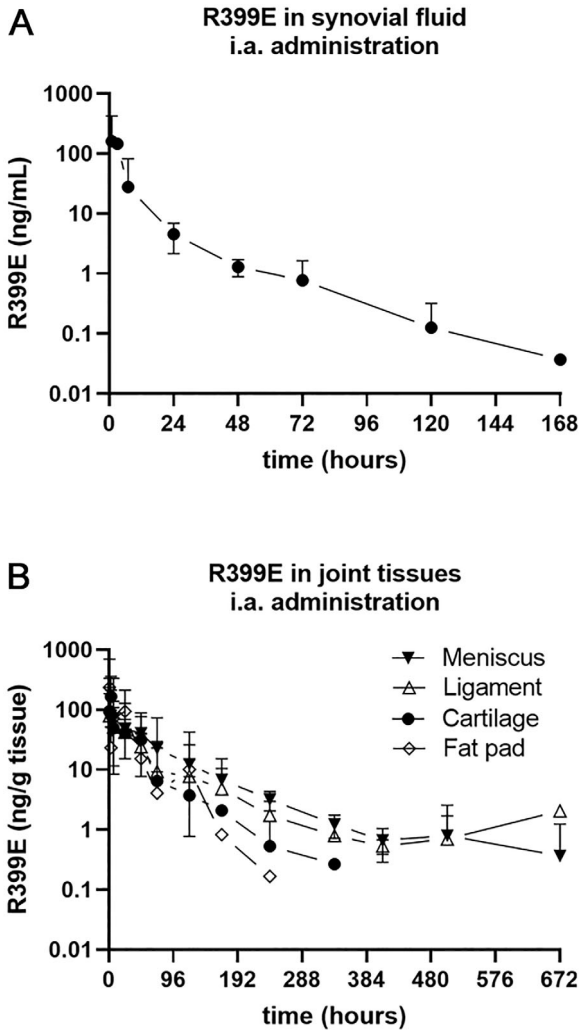

Figure 3. Pharmacokinetics of R399E after intraarticular (IA) injection in rabbits (n = 39 rabbits in a pharmacokinetic study): concentrations of R399E are detectable in synovial fluid up to 7 days and in various joint tissues for 14–28 days. **A**, Concentration of R399E in synovial fluid after single IA administration of 2 μg/joint in rabbits (n = 3 per group) over time. Synovial fluid lavage (SFL) was performed by injecting vehicle then withdrawing the SFL with the same needle. R399E concentrations were measured and then corrected for the dilution that occurred to the synovial fluid. The correction factor was calculated as the ratio between serum and synovial fluid lavage K+ concentration. **B,** R399E meniscus, ligament, cartilage, and fat pad amount (ng/gm tissue) after single IA administration of 2 μg/joint in rabbits (n = 3 per group) over time. Following euthanasia, the injected knee from each animal was collected, frozen, and then pulverized and resuspended using extraction buffer to measure R399E. The highest exposure overall (area under the curve 0–∞) was observed in the meniscus. Concentration levels decreased with time, with quantifiable levels of R399E in the fat pad, cartilage, and both ligament and meniscus up to 240, 336, and ≥672 hours after injection, respectively. Bars show the mean and 95% confidence intervals.

fluid up to 168 hours after injection. The mean terminal half-life was 22.6 hours. In the knee tissues, the highest concentrations of R399E were measured after 1 and 3 hours (Figure 3B). Comparable peak concentrations were found in ligament and meniscus, while the concentration in cartilage was ∼2-fold greater, and the highest concentration was measured in the fat pad. Postpeak tissue concentrations decreased more slowly when compared to serum and synovial fluid, with half-life values of 30.7, 54.4, 60.1, and 83.3 hours in the fat pad, cartilage, ligament, and meniscus, respectively. Tissue exposure (area under the curve [AUC] of 0–∞) was comparable in cartilage, fat pad, and ligament, while higher exposure was observed in the meniscus. Following a single IA administration, R399E was quantifiable in serum only in 2 animals 3 hours after administration and in 1 animal 24 hours after administration, indicating limited systemic absorption (Supplementary Table 1, https://onlinelibrary.wiley.com/doi/10.1002/art.42343). All baseline values were below the limit of quantification (LOQ), indicating no measurable systemic levels of GDF5. Therefore, all values above LOQ reflect levels of R399E.

Effects of R399E on symptomatic and structural measures in a rabbit model of OA. We applied an observer-independent static weight-bearing test and determined deviations from symmetrical weight bearing of the hind limbs (incapacitance) as voluntary expressions of unilateral knee joint–related pain. Intensive training and cage-free housing enabled unbiased contact-free measurements in relaxed animals. Before induction of OA, rabbits put similar load on both hind limbs, and mean weight bearing was around 50% in all groups. One week after induction of OA, rabbits reduced the load on the operated limb resulting in incapacitance of 32% to 35%. Before and 1 week after induction of OA, differences between groups in incapacitance were small and nonsignificant (Figure 4A and Table 1). Six hours after the first injection, R399E significantly improved incapacitance compared to vehicle at all doses (Figure 4A and Table 1). This improvement remained significant for all doses throughout the subsequent measures (except for 0.6 μg R399E at 3 weeks) (Figure 4A and Table 1).

Based on AUC and considering all time points after the first injection, all 3 doses of R399E significantly improved incapacitance compared to vehicle (Figure 4B). Assuming a mean incapacitance in vehicle-treated animals and equal weight distribution as 0% and 100%, respectively, the percentage effect size of R399E based on AUC was 39.4% in the 0.6-μg dose group, 59.4% in the 6-μg dose group, and 37.9% in the 60-μg dose group (Figure 4B). The 6-μg dose of R399E was most efficacious and statistically superior to the 0.6-μg ($P < 0.01$) and 60-μg ($P < 0.05$) doses of R399E. Using gross morphology, the cartilage surface of femoral condyles was mapped with a score ranging from 0 (intact cartilage) to 5 (bony area), and the percentage articular surface for each score was determined. With 6 μg R399E, the percentage surface of medial femur that scored 3 (cracks and fissures) was significantly reduced by 59.5% (Figure 4C), while the percentage surface area of intact cartilage

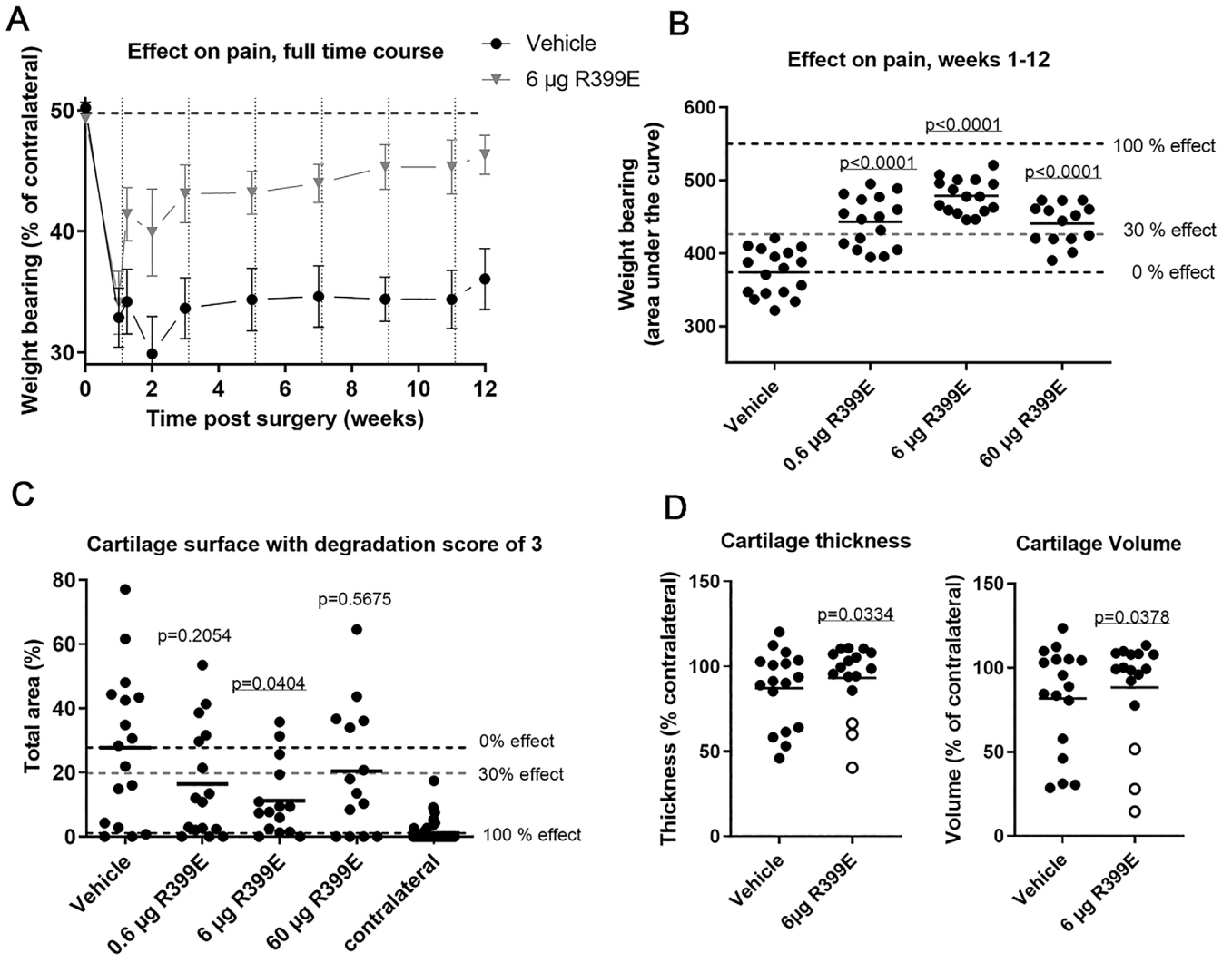

Figure 4. Effects of R399E on symptomatic and structural measures of OA in rabbits after anterior cruciate ligament transection and partial meniscectomy (OA study 1, n = 65 rabbits). Animals received vehicle or 0.6, 6, or 60 µg R399E intraarticular (IA) injection every 2 weeks starting 1 week after surgery (n = 16 in R399E treatment group, n = 17 in vehicle group). **A**, Weight bearing for the vehicle and 6 µg R399E groups. Bars show the mean and 95% confidence intervals. Injections are indicated by dashed lines. **B**, Area under the curve for weight bearing from weeks 1 to 12. *P* values were determined by one-way ANOVA followed by Dunnett's test. 0% effect = mean of vehicle, 100% effect = equal weight distribution. **C**, Gross morphology evaluation of the knee joints. Percentage cartilage area with cartilage degradation score of 3 (cracks and fissures) is shown. *P* values were determined by one-way ANOVA and Dunnett's test. 0% effect = mean of vehicle, 100% effect = total area of 0. Two rabbits from the 60-µg R399E group had to be euthanized due to animal welfare reasons; data are shown for the remaining 14 rabbits. **D**, Cartilage volume and thickness assessed by micro–computed tomography for vehicle (n = 17) and 6-µg R399E groups (n = 13; 3 outliers excluded, shown as open circles). *P* values were determined by Student's *t*-test. In **B–D**, symbols represent individual animals, and bars show the mean per group. *P* values are underlined when $P < 0.05$. See Figure 1 for other definitions.

in medial femur exhibited a nonsignificant trend for improvement by 23.7% compared to vehicle (data not shown). In addition, we quantified cartilage using an automated and observer-independent contrast-enhanced micro-CT method and showed significantly increased cartilage volume and thickness in the femoral weight-bearing areas of rabbit that received 6 µg R399E as compared to vehicle-treated animals (Figure 4D). Based on these results, 6 µg R399E significantly improved both structural and symptomatic measures in the rabbit model of OA with anterior cruciate ligament and partial resection of the medial meniscus.

In a second study using the same model with fewer animals per group (n = 11–13/group), we compared the effect size and dynamic to that of compounds of known clinical efficacy on OA pain. Again, 6 µg of R399E significantly improved incapacitance at the time of first measurement (6 hours after first IA injection) and throughout the study (*P* for AUC < 0.01) (Supplementary Figures 6A and B, https://onlinelibrary.wiley.com/doi/10.1002/art.42343). Repeated doses of 60 µg R399E but not 0.6 µg R399E were also significantly effective (*P* for AUC < 0.01). R399E induced a dose-dependent trend in improved

Table 1. Weight bearing analysis before and during treatment with R399E in the rabbit model of OA*

	R399E 0.6 μg		R399E 6 μg		R399E 60 μg	
	Mean difference (95% CI) compared to vehicle	Adjusted *P*	Mean difference (95% CI) compared to vehicle	Adjusted *P*	Mean difference (95% CI) compared to vehicle	Adjusted *P*
Before surgery	1.0 (−2.73, 4.67)	0.8755	0.9 (−2.79, 4.60)	0.8932	0.48 (−3.35, 4.30)	0.9836
Baseline (1 week after surgery)	−1.6 (−5.30, 2.08)	0.6108	−1.23 (−4.93, 2.46)	0.7758	−1.62 (−5.45, 2.20)	0.6302
6 hours	−5.8 (−9.54, −2.15)	0.0006	−7.2 (−10.93, −3.54)	<0.0001	−7.7 (−11.49, −3.84)	<0.0001
2 weeks	−9.2 (−12.85, −5.46)	<0.0001	−10.0 (−13.72, −6.33)	<0.0001	−10.9 (−14.75, −7.10)	<0.0001
3 weeks	−3.4 (−7.07, 0.31)	0.0819	−9.5 (−13.15, −5.76)	<0.0001	−6.3 (−10.08, −2.43)	0.0004
5 weeks	−5.5 (−9.18, −1.79)	0.0015	−8.8 (−12.54, −5.15)	<0.0001	−7.7 (−11.57, −3.71)	<0.0001
7 weeks	−5.5 (−9.23, −1.84)	0.0013	−9.37 (−13.05, −5.66)	<0.0001	−7.5 (−11.37, −3.71)	<0.0001
9 weeks	−7.7 (−11.40, −4.01)	<0.0001	−10.93 (−14.62, −7.23)	<0.0001	−6.3 (−10.13, −2.47)	0.0003
11 weeks	−7.9 (−11.6, −4.18)	<0.0001	−11.0 (−14.64, −7.25)	<0.0001	−6.1 (−9.92, −2.26)	0.0006
12 weeks	−5.9 (−9.64, −2.25)	<0.0001	−10.3 (−13.98, −6.59)	<0.0001	−4.9 (−8.71, −1.05)	0.0078

* Groups treated with R399E were compared to those treated vehicle using two-way analysis of variance (Dunnett's multiple comparisons test). Weight bearing was measured prior to surgery, 1 week following surgery immediately before first intraarticular injection of R399E (baseline), 6 hours after the first injection, and then at 2, 3, 5, 7, 9, 11, and 12 weeks after surgery.

cartilage degradation score without reaching statistical significance with the reduced number of animals per group (Supplementary Figure 6C). The fifth group received an initial single human-equivalent dose of triamcinolone IA in week 1 postsurgery. This transiently improved incapacitance by 21.6% 6 hours postinjection without reaching statistical significance (Supplementary Figure 6A), and no further effects on incapacitance were observed at weeks 2, 3, and 5 (Supplementary Figures 6A and B). In weeks 5 and 9 after surgery, we injected the same animals intravenously with a human-equivalent dose of an anti-NGF antibody, which produced steady improvements in incapacitance, reaching the same level achieved by 6 μg R399E in week 11 (Supplementary Figure 6A). Of note, the cartilage degradation score was significantly impaired in the group treated with triamcinolone followed by anti-NGF antibody compared to the vehicle- and R399E- treated groups (Supplementary Figure 6C).

Effects of R399E on symptomatic and structural measures in an ovine model of OA. We used the medial meniscus transection model in sheep (25) to investigate R399E in a large animal model of OA (n = 35). Structural improvements were first determined by quantitative scoring of histologic sections, which were obtained from the 4 compartments (medial and lateral tibia and femur) of the operated joint and scored using a modified Mankin score. When the histologic scores were added together, there was a significant reduction in damage in animals receiving 12 μg and 120 μg R399E per joint compared to vehicle controls (Figure 5A). Gross morphologic evaluation for surface damage and osteophyte production in the operated joint revealed a reduction of damage in animals receiving 12 μg and 120 μg R399E per joint (Figure 5B), which was statistically significant ($P = 0.02$) in the 120-μg dose group. Furthermore, radiographs were obtained from all operated hind limbs postmortem.

There was a trend toward a reduction in radiographic K/L score in animals treated with 120 μg R399E per joint (Figure 5C). A possible symptomatic benefit was tested as an exploratory end point via a force plate analysis measuring the weight load on the hind legs. Weight bearing on the operated and unoperated hindlimb was measured prior to surgery and weekly or every other week thereafter (Figure 5D); animals treated with R399E had improved weight bearing on the operated limb over the course of the experiment. The most efficacious R399E dose was 120 μg per joint, with the benefit (compared to vehicle only) evident as early as 3 weeks after the first injection and maintained until week 9 (Figure 5D), without reaching statistical significance (Figure 5E). R399E treatment resulted in a structural benefit and a trend toward symptomatic improvement in an ovine model of OA.

DISCUSSION

The main objective of the present study was to evaluate the potential of R399E as a disease-modifying OA drug (DMOAD). We demonstrated that R399E positively impacted symptomatic as well as structural end points, which fulfills a key aspect of the current expectations for OA therapies among health agencies such as the US Food and Drug Administration, namely that DMOADs show impact on structural end points together with impact on outcomes that matter, i.e., how the patient feels, functions, or survives (26).

We first conducted a series of investigations in vitro to demonstrate anabolic efficacy which may contribute to R399E's potential to improve cartilage structure in patients with knee OA. The main constituents of cartilage matrix are proteoglycans and type II collagen (27), and R399E had positive impact on both: in 3-D cultures of human OA chondrocytes, R399E induced significant and concentration-dependent accumulation of GAG, an

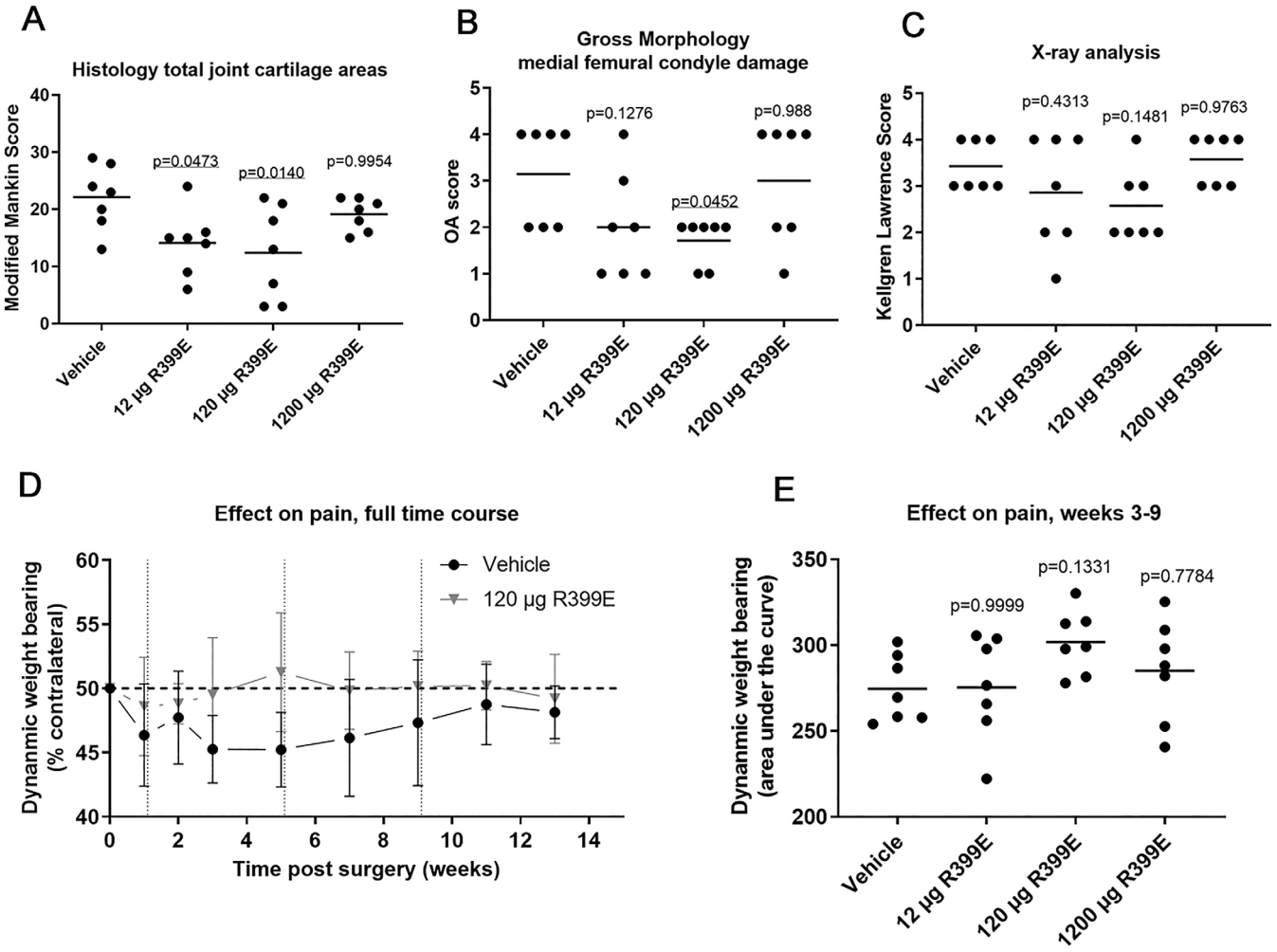

Figure 5. Effects of R399E on symptomatic and structural measures of OA in sheep after medial meniscus transection in the left knee (n = 35). One week after surgery, animals were treated with vehicle or with 12, 120, or 1,200 µg R399E administered intraarticularly every 4 weeks (n = 7 animals/treatment group). **A**, After 13 weeks, animals were euthanized, and knees were prepared for histologic analysis and scored with a modified Mankin score. **B**, Gross morphology assessment of the operated joint was made prior to histology. **C**, Radiographs were obtained postmortem. **D**, Dynamic incapacitance was measured at several time points. Weight bearing is expressed as percentage of the contralateral knee. The horizontal dashed line represents the 50:50 distribution of the pressure load and thus voluntary equal loading of both hind limbs. Bars show the mean and 95% confidence intervals. **E**, The area under the curve was calculated for weeks 3 to 9. A one-way ANOVA followed by a Dunnett's test to correct for multiple comparisons was performed to compare the treatment groups to the vehicle. In **A–C** and **E**, symbols represent individual animals, and bars show the mean per group. *P* values are underlined when $P < 0.05$. See Figure 1 for other definitions.

indicator of proteoglycan production, and increased release of PRO-C2, a propeptide of type II collagen which is released during cartilage formation and collagen synthesis (28). Moreover, R399E increased the release of PRO-C6, indicative of enhanced type VI collagen formation, which is a major component of the chondrocyte pericellular matrix. R399E also induced chondrocyte proliferation possibly resulting from a direct proliferative action of R399E or as an indirect consequence of the increased formation of type VI collagen which has been shown to stimulate chondrocyte proliferation (29). The anabolic responsiveness upon R399E administration varied substantially among donors: although all anabolic readouts were significantly improved overall, 1 of the 5 donors showed a minimal response. This variability in the chondrocyte response reflects the heterogeneity of OA phenotypes (30) and is frequently observed when working with human-derived OA material (31,32).

To be efficacious after IA delivery, temporal tissue exposure to R399E must be sufficient. Using 3-D constructs of chondrocytes, we found that intermittent exposure to R399E (i.e., at weeks 1 and 5 of an 8-week study or at weeks 1, 5, and 9 of a 20-week study) was sufficient to increase GAG and type II collagen content. This was confirmed in vivo in the sheep study where efficacy on structural readouts had been achieved with monthly injections. Following a single 2-µg IA injection, concentrations of R399E were detectable in cartilage for up to 14 days and in menisci for up to 28 days. Though synovial fluid concentrations

of R399E decreased rapidly, measurable levels of R399E remained, particularly in the ligament and meniscus possibly due to R399E binding to the proteoglycans present in knee tissues. Moreover, we have solved the crystal structure of R399E and found a salt bridge between Glu 399 and Arg 438 in both R399E monomers, which is enabled by the R399E mutation. This additional structural stabilization may further contribute to achieve sufficient tissue exposure to R399E after IA injection. These data on PK and stability properties of R399E together with significant efficacy data obtained with intermittent exposure suggest that with 3 IA injections over 6 months, structural benefit is achievable in patients with OA. Since IA injections are invasive and associated with risks, e.g., infection or misinjection, it is important to note that 3 IA injections every 6 months appears to be acceptable for patients, as many are already practiced with certain approved hyaluronic acid preparations (33).

R399E significantly improved pain-related symptoms in a rabbit model of OA after IA injection. Since systemic exposure following IA injection was minimal, it is reasonable to assume that pain was reduced by mechanisms localized within the knee joint. In rabbits, pain reduction was seen as early as 6 hours after the first IA injection, which may indicate that R399E has directly inhibited the excitability of receptive endings of nociceptive neurons in joint tissues, although we are not aware of data supporting this assumption. Conversely, R399E may have reduced localized OA-related inflammation leading to an indirect reduction in neuronal excitability. Indeed, we could demonstrate that R399E reduced the release of IL-1β and PGE_2 in cartilage/synovium coculture, the release of IL-1β and IL-6 from synoviocytes, and the release of PGE_2 from meniscal cells. IL-1β, IL-6, and PGE_2 are known to be able to sensitize nociceptive nerve fibers (34,35); synovium and meniscus but not cartilage are densely innervated, and intraarticular activation of these nerve fibers is considered to be essential for OA pain.

Our results are in accordance with the reported antiinflammatory effects of BMP7 and GDF5, which act as R399E through the BMPR1A, BMPR1B, and BMPR2 (20,36,37). For instance, it was demonstrated that BMP7 polarizes THP-1 cells (human monocyte cell line) into antiinflammatory macrophages leading to an increase of antiinflammatory factors (IL-10, IL-1ra) and a reduced production of the inflammatory cytokines IL-6, TNF, and monocyte chemotactic protein 1 (36). These results were confirmed in vivo by the same research group: in an arteriosclerosis animal model, BMP7 administered intravenously was found to reduce neutrophile infiltration in arteries and to induce M2 macrophages. Circulating IL-10 and IL-1ra were increased, while inflammatory cytokines were decreased. Others observed that overexpression of GDF5 in nucleus pulposus cells reduced TNF, IL-1β, PGE_2, and nitric oxide production and partially restored type II collagen and aggrecan production after lipopolysaccharide stimulation (37).

While the antiinflammatory properties of R399E are probably essential for the pain reduction observed in vivo, it is unknown whether R399E has any direct inhibitory effects on nociceptive neurons. Interestingly, antinociceptive efficacy has explicitly been described for BMP7 (38): it has been shown that BMPR1A and BMPR1B are expressed in the central nervous system and that intracerebral treatment with BMP7 in a rat or mouse sciatic nerve injury model slowed down allodynia development and attenuated its severity. Furthermore, the authors demonstrated that this antiallodynic effect relied on an endogenous opioid-mediated pain relief mechanism. The question of whether a similar mechanism operates with R399E in the animal models of OA is a subject for future investigations.

Limitations of this study include whether our findings can predict whether R399E will be effective in patients with OA. We have demonstrated that R399E is effective in a variety of different in vitro assays predominantly using human tissues as well as in animal models of OA in 2 different species, but predictivity is a general issue for in vitro assays and animal models and is especially pertinent for OA. Indeed, to determine the general predictivity of our assays using clinically effective compounds is very challenging, as there is no approved DMOAD available and only very few clinical candidates have shown efficacy in patients. We have used 2 in vivo models in which knee OA developed as a consequence of surgically induced joint instability. These models have limitations in predicting the most appropriate indication for R399E. Therefore, future in vivo studies could map out more specifically the potential of R399E to treat gradually progressing OA, e.g., in a canine model of spontaneous OA, or to treat chondral defects, e.g., in an ovine model of cartilage injury.

Another limitation of our study is that it provided no information on the duration of action of single IA injections of R399E. We observed significant benefit on a pain-related measure as early as 6 hours after the first injection, and this benefit remained observable 2 weeks later, prior to the next injection. However, we do not know the potential duration of this benefit. Similarly, monthly injections in sheep resulted in significant effects on structural measures, but it is not clear whether an IA injection every second or third month would have been sufficient. A second question that remains to be answered in future studies is the duration over which R399E remains biologically active following IA injection when compared to the 1-month detectability of R399E observed here.

In the current study, only female rabbits (and sheep) were used. This enabled us to house rabbits in a single large colony but is certainly a limitation that should be addressed in future preclinical models, e.g., in dogs, in which both male and female animals could be included.

In conclusion, the tolerability and efficacy of R399E in patients with knee OA will have to be mapped out in future clinical trials, which appear justified based on the preclinical data outlined here.

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AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Gigout had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Gigout, Werkmann, Menges, Brenneis, Henson, Cowan, Musil, Thudium, Gühring, Michaelis, Kleinschmidt-Doerr.

Acquisition of data. Gigout, Werkmann, Menges, Brenneis, Henson, Cowan, Musil, Thudium, Kleinschmidt-Doerr.

Analysis and interpretation of data. Gigout, Werkmann, Menges, Brenneis, Henson, Cowan, Musil, Thudium, Gühring, Michaelis, Kleinschmidt-Doerr.

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Merck KGaA facilitated the study design, provided writing assistance for the manuscript, and reviewed and approved the manuscript prior to submission. The authors independently collected the data, interpreted the results, and had the final decision to submit the manuscript for publication. Writing assistance was provided by Bioscript Group. Publication of this article was not contingent upon approval by Merck KGaA.

ADDITIONAL DISCLOSURE

Author Thudium is an employee of Nordic Bioscience A/S.

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