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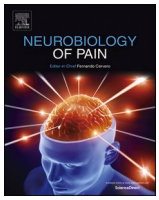

Monoclonal antibodies against GFRα3 are efficacious against evoked hyperalgesic and allodynic responses in mouse join pain models but, one of these, REGN5069, was not effective against pain in a randomized, placebo-controlled clinical trial in patients with osteoarthritis pain

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ABSTRACT

The artemin-GFRα3 signaling pathway has been implicated in various painful conditions including migraine, cold allodynia, hyperalgesia, inflammatory bone pain, and mouse knees contain GFRα3-immunoreactive nerve endings. We developed high affinity mouse (REGN1967) and human (REGN5069) GFRα3-blocking monoclonal antibodies and, following *in vivo* evaluations in mouse models of chronic joint pain (osteoarthritic-like and inflammatory), conducted a first-in-human phase 1 pharmacokinetics (PK) and safety trial of REGN5069 (NCT03645746) in healthy volunteers, and a phase 2 randomized placebo-controlled efficacy and safety trial of REGN5069 (NCT03956550) in patients with knee osteoarthritis (OA) pain. In three commonly used mouse models of chronic joint pain (destabilization of the medial meniscus, intra-articular monoiodoacetate, or Complete Freund's Adjuvant), REGN1967 and REGN5069 attenuated evoked behaviors including tactile allodynia and thermal hyperalgesia without discernably impacting joint pathology or inflammation, prompting us to further evaluate REGN5069 in humans. In the phase 1 study in healthy subjects, the safety profiles of single doses of REGN5069 up to 3000 mg (intravenous) or 600 mg (subcutaneous) were comparable to placebo; PK were consistent with a monoclonal antibody exhibiting target-mediated disposition. In the phase 2 study in patients with OA knee pain, two doses of REGN5069 (100 mg or 1000 mg intravenous every 4 weeks) for 8 weeks failed to achieve the 12-week primary and secondary efficacy endpoints relative to placebo. In addition to possible differences in GFRα3 biology between mice and humans, we highlight here differences in experimental parameters that could have contributed to a different profile of efficacy in mouse models versus human OA pain. Additional research is required to more fully evaluate any potential role of GFRα3 in human pain.

Introduction

The glial-cell line derived neurotrophic factor (GDNF) family of receptors and their associated ligands, a subset of the TGF-β superfamily of proteins, have important neurodevelopmental functions, supporting the differentiation and survival of peripheral and central neurons (Durbec et al., 1996; Treanor et al., 1996; Jing et al., 1997; Baloh et al., 1998; Naveilhan et al., 1998; Trupp et al., 1999; Sariola et al., 2003). GDNF family receptors (of which five subtypes have been identified, GFRα1–4 and GFRαL), show high affinity for their respective ligands and display

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differential anatomic localization; however, all are glycosylphosphatidylinositol-linked proteins, which lack intracellular domains. Signal transduction is accomplished when receptor and ligand form a tripartite complex with the rearranged during transfection (RET) receptor tyrosine kinase, which contains a cytoplasmic signaling domain (Durbec et al., 1996; Treanor et al., 1996; Jing et al., 1997; Baloh et al., 1998; Naveilhan et al., 1998; Trupp et al., 1999; Sariola et al., 2003).

In addition to their involvement in neurodevelopment, there is evidence to suggest a potential role for the GDNF family receptor alpha-3 (GFRα3) and its only known high affinity ligand, artemin, in nociceptive signaling (Malin et al., 2006; Nencini et al., 2018). In mouse, GFRα3 is preferentially expressed on sensory and sympathetic neurons of the peripheral nervous system (Honma et al., 2002), which we have also observed using a lacZ reporter inserted into the *gfra3* locus (Supplemental Fig. 1). In adult humans, GFRα3 is localized to dorsal root, trigeminal and sympathetic ganglia, as well as peripheral nerves (Bespalov et al., 2007). Local administration or injection of artemin in rodents to the paw, face, or tongue causes hyperalgesic responses (Elitt et al., 2006; Malin et al., 2006; Elitt et al., 2008; Thornton et al., 2013), and localized increases in artemin levels have been reported in human patients with atopic dermatitis (Murota et al., 2012) and in burning tongue syndrome (Shinoda et al., 2015). Artemin may also play a role in inflammatory pain, because inhibition of artemin signaling with neutralizing antibodies attenuates nociceptive responses to intra-plantar Complete Freund's Adjuvant (CFA) (Thornton et al., 2013), tongue inflammation (Shinoda et al., 2015), intra-articular monoiodoacetate (MIA)-induced arthritis (Minnema et al., 2022) and experimentally-induced interstitial cystitis in rodents (DeBerry et al., 2015). In a 'first in human' clinical trial testing both intravenous (IV) and subcutaneous (SC) administration of recombinant artemin (neublastin), IV administration was associated with temperature sensation disruptions (most commonly feeling hot) and pruritus (Rolan et al., 2015), and in a placebo-controlled study of neublastin in patients with painful lumbosacral radiculopathy, there was no clear dose–response relationship for pain reduction (Backonja et al., 2017).

The GFRα3-artemin signaling pathway has been implicated in various painful conditions including migraine, cold allodynia, hyperalgesia, and inflammatory bone pain (Orozco et al., 2001; Malin et al., 2006; Lippoldt et al., 2013; Shang et al., 2016; Nencini et al., 2018; Minnema et al., 2020; Zhu et al., 2020), but little has been reported on its potential role in musculoskeletal pain such as chronic joint pain. The knee joint is innervated by small peptidergic nociceptive fibers (Mach et al., 2002), which have been reported to express GFRα3 (Nencini et al., 2018). Nerve fiber sprouting has been observed in joints after injection of CFA into mouse knee (Ghilardi et al., 2012), and in bone during bone cancer pain in mice (Jimenez-Andrade et al., 2010). These pathological nerve sprouts can be reduced by inhibition of another growth factor protein, nerve growth factor (NGF) (Jimenez-Andrade et al., 2011; Ghilardi et al., 2012). While there is currently no evidence that GFRα3-artemin signaling is involved in pathological nerve sprouting, it is possible that it is involved in the sensitization of sprouted fibers.

Here, we report a series of studies with high affinity mouse (REGN1967) and human (REGN5069) GFRα3-blocking monoclonal antibodies in three mouse models of chronic evoked joint pain (osteoarthritic-like and inflammatory), a first-in-human phase 1 pharmacokinetics (PK) and safety trial of REGN5069, and a phase 2 randomized placebo-controlled efficacy and safety trial of REGN5069 in patients with pain due to osteoarthritis (OA) of the knee.

Methods

Preclinical studies

Antibody generation and screening

Mouse GFRα3 antibodies were generated by immunizing *gfra3* knockout mice (Supplemental Fig. 1) with mouse GFRα3-myc-myc-His (mmH). Human GFRα3 antibodies were generated by immunizing VelocImmune® mice (Macdonald et al., 2014) with human GFRα3-mouse Fc. Antibodies were isolated and screened for antigen specificity and binding affinity by surface plasmon resonance (Biacore T200), and ability to block artemin-mediated GFRα3 signaling in a luciferase bioassay in HEK293 cells expressing GFRα3 and RET (see Supplemental methods for further details). The anti-mouse GFRα3 and anti-human GFRα3 antibodies with the highest affinities and the lowest IC_{50} values in the blocking assay (designated REGN1967 and REGN5069 respectively) were purified and produced in quantities sufficient for *in vivo* testing.

Animal subjects

Adult C57BL/6 male mice (Jackson Laboratories, Bar Harbor, ME), for evaluating anti-mouse GFRα3 antibodies or adult humanized GFRα3 male and female mice (Supplemental Fig. 1) for evaluating anti-human GFRα3 antibodies, were used for all *in vivo* experiments. Initial experiments were conducted with the anti-mouse antibody in C57BL/6 mice, with a transition to most experiments using the anti-human antibody once humanized GFRα3 mice became available. For most experiments, mice were 10–16 weeks old at initiation of experiments. In the destabilization of the medial meniscus (DMM) model, mice were 6–8 months old because OA is more common in middle to older aged humans and DMM-related joint pathology develops over time. Animals were housed in groups of up to five in a temperature-controlled environment on a 12-hour light/dark cycle with water and standard laboratory chow available *ad libitum*. In the week prior to surgical manipulations, mice were handled and placed in the behavioral testing apparatus for at least 2 h per day to acclimate them to the room and equipment. Animals were additionally acclimated for at least 1 h before every subsequent testing session. Baseline nociceptive responses were obtained after the 1-week acclimation period.

Drug administration

All therapeutic agents were administered to mice by SC injection. Antibodies were diluted in sterile phosphate buffered saline and administered once or twice per week depending on the study. The appropriate isotype antibody (REGN1094 for REGN1967 and REGN1945 for REGN5069) was given to a control group of animals for all *in vivo* experiments. A monoclonal antibody against NGF (REGN475) was used as a positive control for NGF inhibition in the intra-plantar thermal hyperalgesia model.

The non-steroidal anti-inflammatory drug (NSAID) indomethacin (Sigma-Aldrich, St. Louis, MO) was diluted in 0.5% methylcellulose (Sigma-Aldrich) and administered at 1 mg/kg by SC injection three times per week as a comparator for two DMM experiments. Mice in the intra-knee models (CFA and MIA) were treated with phosphate buffered saline or antibodies (REGN1967, REGN5069, REGN1094 or REGN1945 50 mg/kg) 24 h before intra-knee injections, and once weekly thereafter.

Mouse in vivo models and behavioral tests

Efficacy of anti-GFRα3 antibodies was assessed in mice using an intra-plantar model of artemin-induced hyperalgesia and in three different chronic joint pain models. Nociceptive responses included tactile allodynia assessed by measuring paw withdrawal to calibrated von Frey filaments and thermal hyperalgesia evaluated using the Hargreaves Test (Hargreaves et al., 1988) (see Supplemental methods for further details). Each of the chronic pain models involved the right hind limb. Under isoflurane anesthesia (3% isoflurane in oxygen), the limb was shaved, and the skin cleaned with antiseptic while mice were supine, and the knee extended and secured prior to surgical manipulation/intra-articular injection.

Induction of growth factor-induced plantar thermal hyperalgesia. Baseline thermal hyperalgesia was evaluated using the Hargreaves Test before SC

administration of 50 mg/kg REGN1967 (anti-mouse GFRα3 antibody), REGN5069 (anti-human GFRα3 antibody), REGN1094 (isotype control for REGN1967), REGN1945 (isotype control for REGN5069) or REGN475 (anti-NGF antibody). Three days later, mice were placed into a clear plastic restrainer with their hind limbs exposed and recombinant mouse artemin or mouse NGF (0.5 μg in 20 μL saline, both from R&D Systems, Minneapolis, MN) was injected into the plantar surface of the left hind paw. Thermal hyperalgesia was re-evaluated 4 days later.

Induction of OA-like pain by destabilization of the medial meniscus. A 3 mm longitudinal incision was made in the skin over the distal patella to the proximal tibial plateau. An incision was then made in the joint capsule, which was opened to dissect out the fat pad. The medial meniscotibial ligament was cut to free the medial meniscus, which was fully excised (1 mm) to destabilize the knee joint, as described by Glasson et al. (Glasson et al., 2007). Animals were then sutured, recovered, and returned to their home cages. Antibodies (REGN1967, REGN5069, REGN1094, or REGN1945) were administered at 50 mg/kg/week (wk) starting either at the time of the DMM surgery or 16 weeks later and animals were evaluated for tactile allodynia. REGN1967 was evaluated in the DMM model in male mice only, and REGN5069 was evaluated for efficacy in the DMM model in both male and female mice.

Induction of joint pain related to cartilage loss (intra-knee monoiodoacetate). The knee was injected with 30 μL of MIA prepared at 10 mg/mL (monoiodoacetate, Sigma-Aldrich [St Louis, MO], in Adjuvant Incomplete Freund's (Becton, Dickinson and Company, Franklin Lakes, NJ) through the infrapatellar ligament. Animals were recovered and returned to their home cages. Tactile allodynia was evaluated 1, 3, and 5 weeks after MIA. In one experiment, animals were treated with 50 mg/kg/wk REGN5069 or REGN1945 starting the same day as injection of MIA. In a second experiment, animals received 5, 25, or 50 mg/kg/wk REGN5069 or 50 mg/kg/wk REGN1945 starting 2 weeks after MIA, immediately following tactile allodynia measured at 2 weeks.

Induction of inflammatory joint pain (intra-knee CFA). The knee was injected with 30 μL of CFA prepared at 10 mg/mL (*Mycobacterium butyricum* in Adjuvant Incomplete Freund's) through the infrapatellar ligament. An additional 50 μL of CFA (Becton, Dickinson and Company) was injected into the hamstring muscle adjacent to the knee. Animals were recovered and returned to their home cages. Tactile allodynia was evaluated 1, 3, and 5 weeks later, and thermal hyperalgesia 4 weeks after CFA.

Dose-response of REGN5069. The above *in vivo* experiments used high doses of monoclonal antibodies ranging from 30 to 50 mg/kg/wk. Dose-response studies were next conducted with REGN5069 in humanized GFRα3 mice to determine whether lower doses could produce significant attenuation of hyperalgesia in the three shorter-term models. In the acute model of artemin-induced thermal hyperalgesia, pre-treatment with doses of REGN5069 ranging from 0.3 to 30 mg/kg were evaluated for efficacy 4 days after intra-plantar injection. In this model, we added a subthreshold injection of intra-plantar capsaicin (0.5 μg) 24 h after artemin. In our hands, the intra-plantar response to artemin is too variable to reliably detect the small effect sizes induced by low doses of REGN5069. We have found, however, that we can produce a consistent, vigorous and sustained hyperalgesic response by using artemin to enhance a subthreshold dose of capsaicin. Artemin's ability to enhance capsaicin's hyperalgesic effects were first reported in the Davis and Albers' labs (Elitt et al., 2006; Malin et al., 2006). Thermal hyperalgesia was also evaluated in the CFA model of inflammatory joint pain 4 weeks after intra-knee CFA with REGN5069 doses ranging from 1 to 50 mg/kg/wk.

Histology studies. Following the chronic joint pain experiments, animals were euthanized, and ipsilateral knee joints were dissected and prepared for histological evaluation. High-resolution micro-tomographic (μCT) imaging of joints following DMM was used to measure osteocyte volume, osteocyte number, and subchondral bone integrity, and light microscopy of MIA and CFA joints was used to assess cartilage density and joint inflammation as previously described (Choe et al., 2003) (see Supplemental methods for further details).

Phase 1 study of REGN5069 in healthy human volunteers

The phase 1 study (NCT03645746) was a randomized, placebo-controlled double-blind trial. Eligible participants were healthy volunteers, 18–55 years old (inclusive) with body mass index 18–31 kg/m^2 (inclusive) (see Supplemental methods for full inclusion and exclusion criteria). Following a screening period and pre-baseline visit, subjects were randomized to one of seven sequential ascending REGN5069 single dose cohorts (30, 100, 300, 1000, or 3000 mg by IV injection or 300 or 600 mg by SC injection) and admitted for an inpatient clinic stay of up to two nights. Eight subjects were enrolled into each cohort and received either REGN5069 ($n = 6$) or placebo ($n = 2$). Placebo contained the same excipient components as the investigational product except the active REGN5069 protein. Participants were followed-up for up to 36 weeks (Supplemental Fig. 2). The primary endpoint was the incidence and severity of treatment-emergent adverse events (TEAEs), as well as other safety variables in participants who received REGN5069 administered IV or SC compared to placebo. Secondary endpoints included the concentration of functional REGN5069 in serum at each time point, anti-drug antibody (ADA) development and assessment of biomarkers (see Supplemental methods for further details).

Phase 2 study of REGN5069 in patients with knee OA pain

The phase 2 study (NCT03956550) was a randomized, double-blind, multi-dose, placebo-controlled efficacy and safety trial in patients ≥ 40 years old with a clinical diagnosis of OA of the knee based on the American College of Rheumatology criteria (Altman et al., 1986) with radiologic evidence of OA (Kellgren–Lawrence [K–L] score ≥ 2) and moderate-to-severe pain in the index joint, defined as having a Western Ontario and McMaster Osteoarthritis Index (WOMAC) pain subscale score of ≥ 4 at screening and baseline visits (see Supplemental methods for full inclusion and exclusion criteria). Patients were randomized 1:1:1 to receive REGN5069 100 mg every 4 weeks (Q4W), REGN5069 1000 mg Q4W, or matching placebo (provided as lyophilized powders in vials for reconstitution) Q4W by IV injections at baseline, week 4, and week 8. Randomization was stratified by K–L category of the index joint at screening and by participation in a device sub-study; the device sub-study results will be reported separately. After the 12-week treatment period and the 24-week follow-up period, an end-of-study phone call to follow-up for joint replacement status was planned approximately 52 weeks after the first dose of study drug. The study was prematurely terminated due to lack of efficacy before all patients completed the week 52 phone call. The primary endpoint was the change from baseline at week 12 in the WOMAC pain subscale score. Secondary efficacy endpoints included change in WOMAC total score, physical function, and stiffness subscale scores, and in the Patient Global Assessment (PGA) score. Safety and tolerability of REGN5069 compared with placebo, concentration of functional REGN5069 in serum, ADA development and assessment of biomarkers were included as secondary objectives (see Supplemental methods for further details).

Statistical analysis

Animal studies

Thermal hyperalgesia: Using pilot results from artemin or NGF injections at a power level of 0.80 and $\alpha = 0.05$, we calculated that we would need eight animals to detect a return to baseline latencies to withdraw with antibody treatment. We used sample sizes of 7–10

animals because these experiments were designed to detect full blockade of the growth factor response, thus minimizing animal use by selecting just over the smallest recommended sample size from the power analysis.

Tactile allodynia: Each animal's mean withdrawal pressure was analyzed using two-way mixed factorial analysis of variances (ANOVA) (time × treatment) with α set at 0.05. Significant interactions or main effects of treatment were probed using Tukey *post hoc* tests. Using changes in tactile allodynia in naïve versus DMM animals measured at 16 weeks after surgery, and a power level of 0.80 and $\alpha = 0.05$, we calculated that we would need six animals to detect a 50% return to control levels after antibody treatment. We used sample sizes of 8–10 animals to ensure adequate power given the long duration of these experiments (28 weeks).

Intra-knee CFA experiments were powered for the most variable behavioral measure, tactile allodynia. Using pilot results from saline versus CFA injections at a power level of 0.80 and $\alpha = 0.05$, we calculated that we would need six animals to detect a 50% return to control levels after CFA injection. We used sample sizes of 7–8 animals to ensure adequate power in this 6-week invasive study.

μCT data and joint inflammation scores were analyzed using one-way independent groups ANOVA to compare treatment groups, with α set at 0.05. Significant main effects of treatment were probed using Tukey *post hoc* tests. Cartilage density scores were analyzed using an independent groups *t*-test to compare the isotype control versus anti-GFRα3 groups, with α set at 0.05.

Human studies

For the phase 1 and 2 studies of REGN5069 in healthy subjects and patients with knee OA pain, safety variables, PK parameters, and immunogenicity variables were summarized descriptively. No formal comparisons and testing against the placebo group or between different dose cohorts were performed for the phase 1 study. The primary and secondary efficacy endpoints in the phase 2 study were analyzed using a multiple imputation approach with a mixed-effects model for repeated measures based on the full analysis set (see Supplemental methods for further details).

Study approval

All *in vivo* experimental procedures in mice were approved by Regeneron Pharmaceuticals, Inc.'s Animal Care and Use Committee and researchers were blinded to the group assignments of the animals. The phase 1 and phase 2 human studies were conducted in accordance with international guidelines including the Declaration of Helsinki and Council for International Organizations of Medical Sciences International Ethical Guidelines, applicable International Conference on Harmonisation Good Clinical Practice Guidelines, the Council for International Organizations of Medical Sciences International Ethical Guidelines, and all applicable laws and regulations. The clinical study protocols, informed consent forms, investigator brochures, and all protocol amendments were approved by the study sites' institutional review boards/ethics committees. All participants provided written informed consent.

Results

In vitro and in vivo animal studies

Antibody generation and screening

Immunization of VelocImmune® mice with human GFRα3-mFc produced high affinity blocking antibodies; however, none of them cross-reacted with mouse GFRα3. Mouse surrogate antibodies against GFRα3 were produced by immunizing GFRα3 knockout mice with mouse GFRα3mmH protein to break tolerance to the 'self' protein GFRα3. After completion of a similar screening, two high affinity mouse blocking antibodies against GFRα3 were identified. The best human (REGN5069) and mouse (REGN1967) antibody each showed high affinity for GFRα3 by Biacore (Table 1 and Supplemental Fig. 3), and both were potent blockers of artemin signaling in a luciferase blocking assay using a HEK293 cell-line with stable expression of GFRα3 and RET (Supplemental Fig. 4). Interestingly, while many of the screened antibodies blocked the binding of GFRα3 to artemin alone in blocking assays, some did so without the addition of RET; however, both selected antibodies required the addition of RET to achieve blockade (data not shown), suggesting that these antibodies might be preferential for inhibition of the signaling complex, at least *in vitro*.

Efficacy of REGN1967 and REGN5069 in the mouse intra-plantar growth factor model

REGN1967 and REGN5069 completely blocked thermal hyperalgesia induced by artemin in the Hargreaves Test at 50 mg/kg SC (Fig. 1A–B). To determine whether the hyperalgesic effects of artemin could be mediated by downstream NGF, an anti-NGF antibody (REGN475) was also administered in this model and was found to have no effect. The inhibitory effects of the anti-GFRα3 antibodies were specific to the hyperalgesic effects of artemin, because neither the mouse nor human antibody inhibited thermal hyperalgesia induced by intra-plantar injection of NGF (Fig. 1C–D). A monoclonal antibody against NGF (REGN475) blocked NGF-induced hyperalgesia (Fig. 1C–D), suggesting that artemin signaling through GFRα3 does not lie directly downstream of NGF's hyperalgesic signaling pathway.

Efficacy of REGN1967 and REGN5069 in the mouse DMM model

Both REGN1967 and REGN5069 produced significant efficacy against tactile allodynia in the DMM model of OA-like pain in adult male mice (Fig. 2A–B), even when the allodynia was refractory to NSAIDS (Fig. 2A). This efficacy was observed with the anti-mouse GFRα3 whether it was administered immediately after the DMM surgery or delayed until 16 weeks later, after the allodynic state was reliably established (Supplemental Fig. 5). Early administration of anti-GFRα3 did not prevent the development of the allodynic state, because tactile allodynia emerged upon withdrawal of antibody treatment. Treatment with anti-GFRα3 antibodies did not affect osteophyte formation. No consistent differences were observed between sexes in efficacy of REGN5069; however, efficacy in males at a single time point was significantly lower (Supplemental Fig. 6A). μCT imaging showed no difference between treatment groups in osteophyte burden (data not shown), and also no differences between treatment groups in measures of subchondral bone integrity in the region of the tibial plate (Supplemental Fig. 6B–D).

Table 1
Ligand binding properties of anti-human GFRα3 (REGN5069) and anti-mouse GFRα3 (REGN1967) antibodies.

Anti-GFRα3	Test ligand	Biacore kinetic parameters for anti-GFRα3 binding to soluble GFRα3 ectodomain at 37 °C			
		k_a ($M^{-1}s^{-1}$)	k_d (s^{-1})	K_D (M)	$T_{1/2}$ (min)
REGN5069 (Anti-human GFRα3)	Human GFRα3.mmH (monomer)	1.41E + 06	5.57E-04	3.96E-10	21
REGN1967 (Anti-mouse GFRa3)	Mouse GFRα3.mmH (monomer)	1.44E + 06	1.47E-05	1.03E-11	784

Summary of equilibrium dissociation constants (KD) for the interaction of surface-captured anti-human GFRα3 and anti-mouse GFRα3 antibody with monomeric recombinant human GFRα3 or mouse GFRα3 protein respectively.
k_a, association rate constant; k_d, dissociation rate constant; K_D, equilibrium dissociation constant; $T_{1/2}$, dissociative half-life; mmH, myc-myc-His.

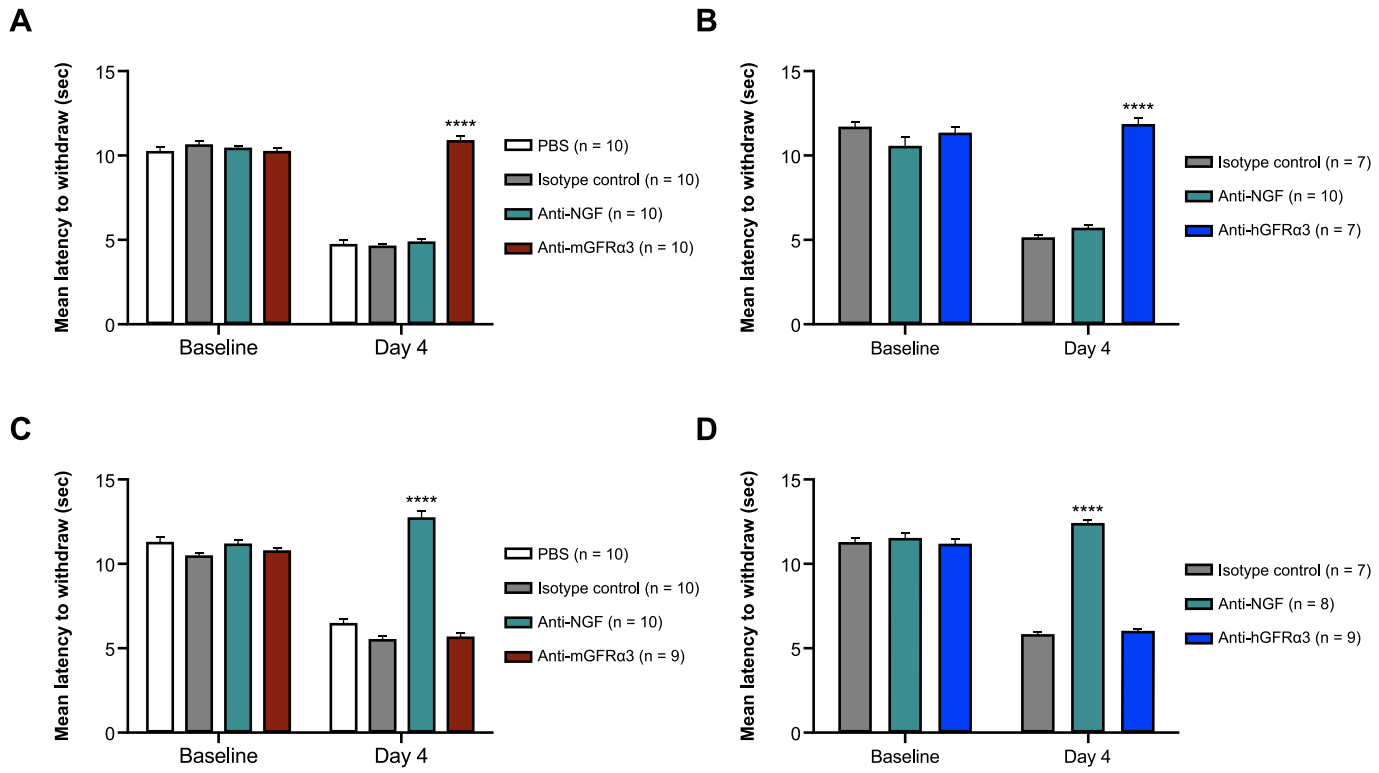

Fig. 1. Blockade of thermal hyperalgesia by REGN1967 and REGN5069 in the mouse intra-plantar growth factor model. Both REGN1967 (A) and REGN5069 (B) monoclonal antibodies at 50 mg/kg/week SC blocked artemin-induced thermal hyperalgesia evaluated by the Hargreaves Test. In contrast, neither the mouse (C) nor the human antibody (D) attenuated NGF-induced thermal hyperalgesia. An anti-NGF antibody (REGN475) was used as a positive control for blockade of NGF-induced thermal hyperalgesia (C and D) to verify that the nociceptive response could be blocked by a cognate antibody. It was also used in the artemin-induced hyperalgesia assay, where it failed to attenuate hyperalgesic responses (A and B). Data shown as mean and standard error of the mean. ****$p < 0.0001$ versus isotype control antibody, Tukey *post hoc* test.

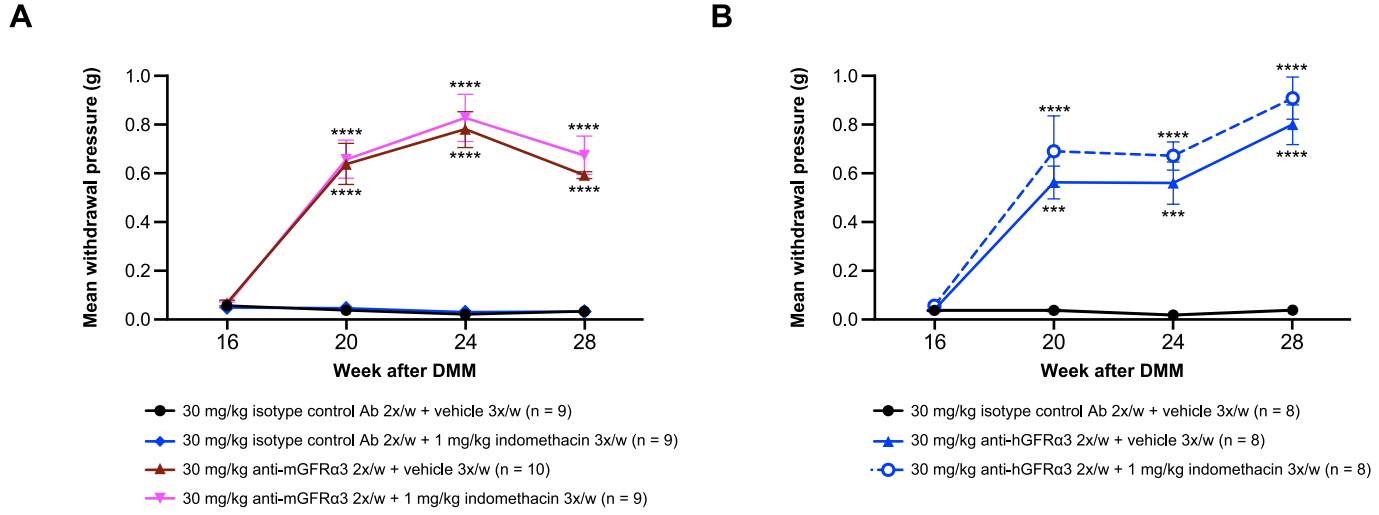

Fig. 2. Attenuation of tactile allodynia by REGN1967 and REGN5069 in the mouse DMM model. Both REGN1967 (A) and REGN5069 (B) significantly attenuated tactile allodynia when administered starting 16 weeks after DMM surgery (after the 16-week von Frey filament evaluation was conducted). The NSAID indomethacin was administered as a comparator and had no effect on DMM-induced allodynia (A), nor did it produce a synergistic or additive effect when given in combination with an anti-GFRα3 antibody (A and B). Data shown as mean and standard error of the mean. ***$p < 0.001$ versus isotype control antibody, Tukey *post hoc* test versus isotype control antibody; ****$p < 0.0001$, Tukey *post hoc* test versus isotype control antibody.

Efficacy of REGN5069 in the mouse MIA model

Intra-knee injection of MIA resulted in chronic pain characterized by tactile allodynia in the ipsilateral paw. Treatment with REGN5069 starting either concurrent with MIA injection (Supplemental Fig. 7A) or 2 weeks after MIA injection (Supplemental Fig. 7B) significantly attenuated allodynia across the experimental timeframe. MIA injection caused substantial cartilage damage in the knee joint, and this damage was not significantly affected by treatment with REGN5069 (Supplemental Fig. 7C–D). Therefore, it appeared that REGN5069 alleviated the nociceptive effects of intra-articular MIA without significantly altering

the underlying chondrocyte pathology.

Efficacy of REGN1967 in the mouse CFA model

Intra-knee injection of CFA resulted in chronic pain characterized by both tactile allodynia and thermal hyperalgesia in the ipsilateral paw. Treatment with REGN1967 significantly attenuated allodynia across the 5 weeks of the experiment (Fig. 3A). The Hargreaves Test revealed that thermal hyperalgesia was also significantly lower with REGN1967 treatment (Fig. 3B). Because the development of nociception is linked to inflammation in many inflammatory pain states, we also evaluated the effect of REGN1967 on joint inflammation induced by CFA. CFA injection caused substantial inflammatory infiltrate in the knee joint and surrounding tissues (Fig. 3C–D), and this inflammation did not significantly differ between treatment groups (Fig. 3D); moreover, all groups administered intra-articular CFA had a similar extent of knee inflammation. Therefore, it appeared that REGN1967 alleviated the nociceptive effects of intra-knee CFA without altering the underlying inflammatory pathology.

Dose-response of REGN5069 in the mouse intra-plantar artemin-sensitized capsaicin and mouse CFA models

Doses of 10 and 30 mg/kg REGN5069 both produced significant efficacy against thermal hyperalgesia in mice pre-treated with intra-plantar artemin and capsaicin, while doses of 0.3 or 3 mg/kg failed to significantly attenuate nociceptive responses (Fig. 4A). In the CFA model of inflammatory joint pain 4 weeks after intra-knee CFA, all doses of REGN5069 (1–50 mg/kg/wk) showed efficacy in attenuating thermal hyperalgesia (Fig. 4B). In the MIA model, significant efficacy against tactile allodynia was achieved for doses 5 mg/kg/wk or higher (Fig. 4C).

Phase 1 'first-in-human' study of REGN5069 in healthy subjects

Participants

In total, 56 healthy subjects were enrolled, of whom > 60% were female and 98% were White; 30 and 12 participants received REGN5069 via IV and SC administration, respectively. Participant disposition is shown in Supplemental Fig. 8. Baseline characteristics are summarized in Supplemental Table 1.

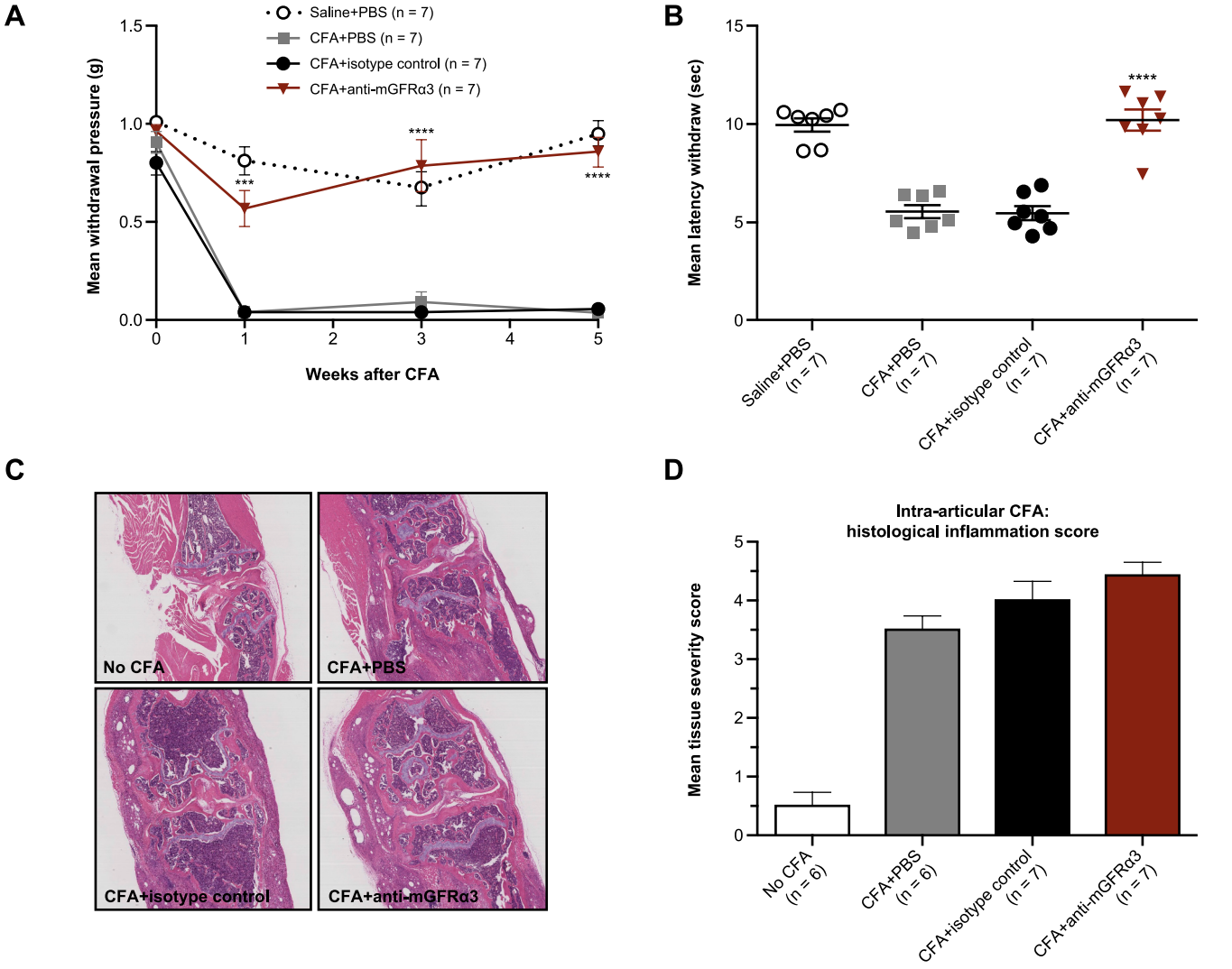

Fig. 3. REGN1967 anti-nociceptive effects in mouse CFA model. REGN1967 at 50 mg/kg/week produced significant anti-nociceptive effects for both (A) tactile allodynia and (B) thermal hyperalgesia after CFA injection into the knee joint. Inflammation was observed by hemotoxylin and eosin staining in the joints of all animals administered CFA (C) and scoring of gross inflammation in these joints revealed no significant differences between treatment groups receiving CFA (D). Data shown as mean and standard error of the mean.***$p < 0.001$, Tukey *post hoc* test versus isotype control antibody; ****$p < 0.0001$, Tukey *post hoc* test versus isotype control antibody.

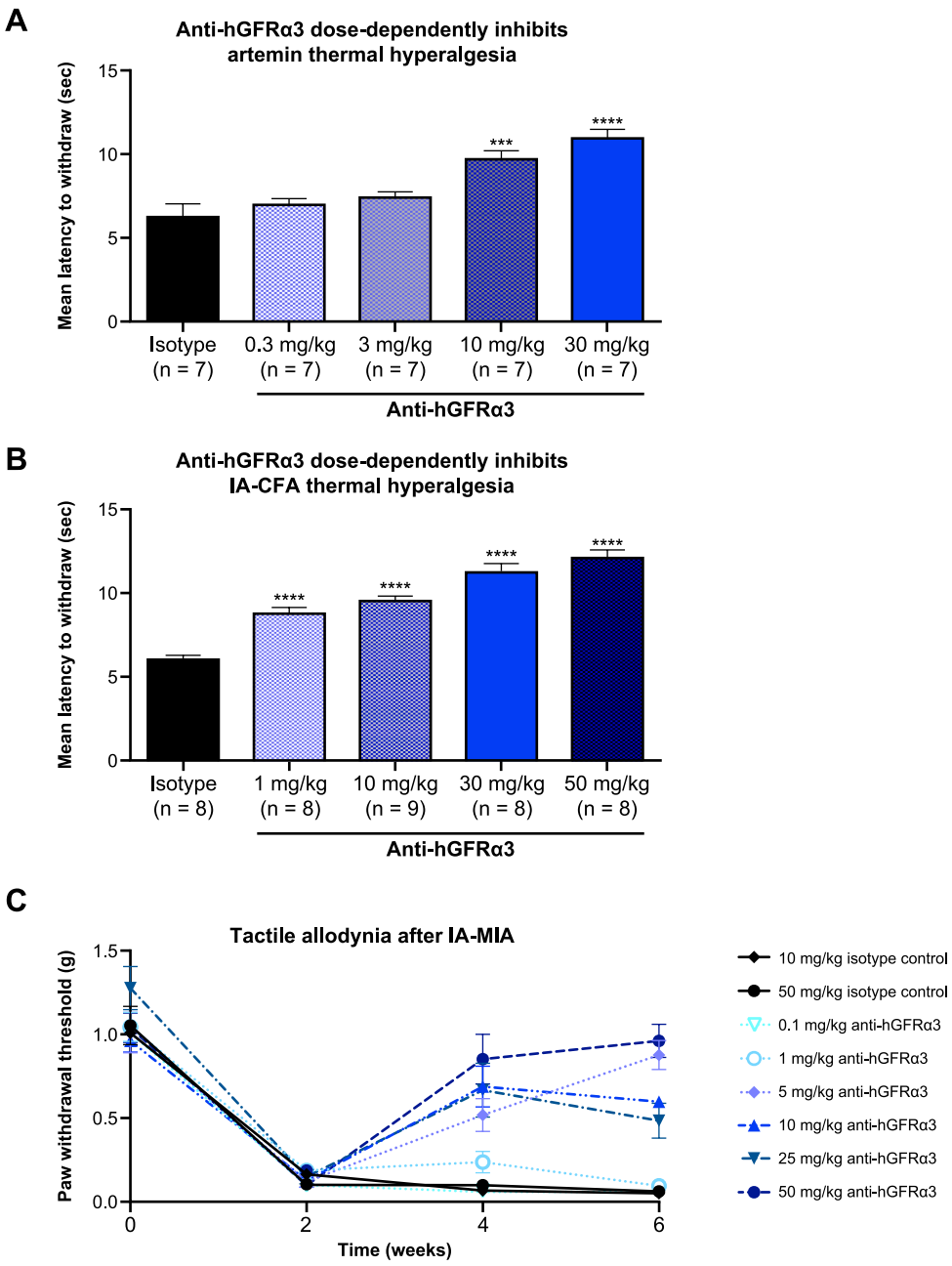

Fig. 4. Dose-response for REGN5069 in attenuating hyperalgesia and allodynia. Dose-response data were generated for REGN5069 using artemin-induced (A), intra-knee CFA-induced thermal hyperalgesia (B), and MIA-induced tactile allodynia as outcome measures (C). Single administration of antibody doses as low as 10 mg/kg produced significant efficacy against artemin-induced nociceptive responses (A), and weekly administration of REGN5069 at doses as low as 1 mg/kg produced efficacy against hyperalgesia after 4 weeks in the intra-knee CFA model (B). Data shown as mean and standard error of the mean. ***$p < 0.001$, Tukey *post hoc* test versus isotype control antibody; ****$p < 0.0001$, Tukey *post hoc* test versus isotype control antibody.

Safety of REGN5069

All 10 participants (100%) in the combined placebo groups and 35 subjects (83%) in the combined REGN5069 groups had at least one TEAE (Supplemental Table 2). No REGN5069-related safety signals were observed when comparing the total REGN5069 group to the total placebo group. There were no serious TEAEs or deaths and most TEAEs were mild or moderate in severity. Two participants (5%) in the combined REGN5069 groups experienced TEAEs of severe intensity: gout-like arthritis in one participant (30 mg IV; considered related to the study drug), and increased creatine phosphokinase in one participant (300 mg IV; considered unrelated to the study drug). Both events resolved. TEAEs reported in ≥ 10% of all REGN5069-treated participants included nasopharyngitis (29% vs 36% for placebo), headache (26% vs 29%) and oropharyngeal pain (24% vs 14%); there were no dose-related trends in TEAEs. In the combined placebo and REGN5069 groups, 36% and 24% of participants respectively had at least one treatment-emergent orthostatic hypotension measurement (Supplemental Table 3). Only two of these events were considered as adverse events (AEs) (one placebo and one REGN5069 100 mg IV subject); both were mild and resolved without treatment. There were no dose-dependent trends or clinically meaningful findings observed from any other safety evaluations performed during the study, including vital signs, 12-lead electrocardiograms, physical and neurological examinations, and orthostatic hypotension measurements.

Pharmacokinetics of REGN5069

Fig. 5 shows mean concentration–time profiles of functional REGN5069 in serum for each dose, consistent with those for a monoclonal antibody exhibiting target-mediated drug disposition. The profiles generally reflect a brief distribution or absorption phase following IV or SC administration, respectively, followed by a slower initial linear elimination phase, and then a more rapid, concentration-dependent elimination phase, presumably mediated by target binding. The target-mediated elimination phase was predominant at concentrations in serum below 4–10 mg/L.

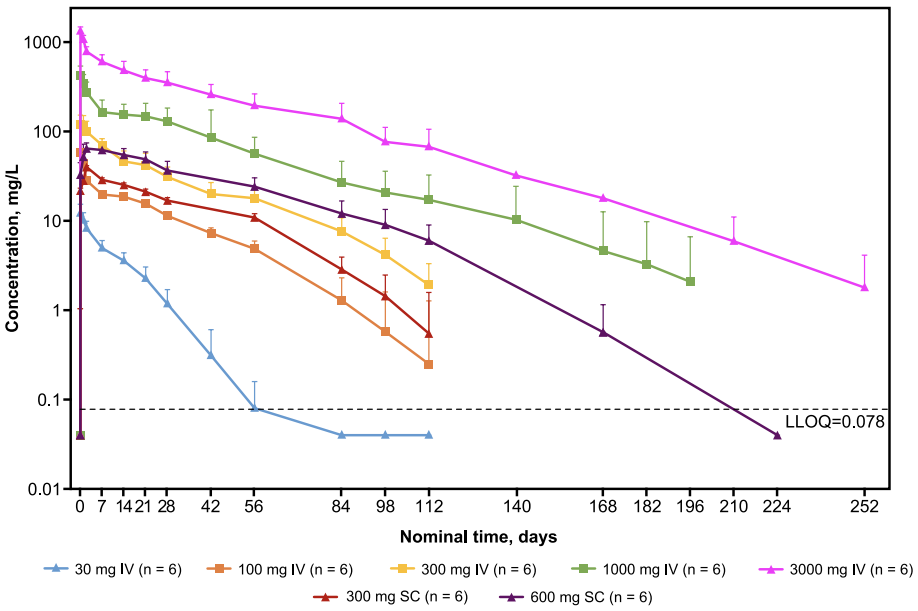

Fig. 5. Concentration-time profiles of functional REGN5069 in serum in the phase 1 study in healthy subjects (PK analysis set). Values are mean + standard deviation. LLOQ, lower limit of quantitation.

PK parameters calculated by noncompartmental analysis are shown in Supplemental Table 4. Consistent with the target-mediated, nonlinear kinetics displayed by the concentration–time profiles, greater than dose-proportional increases in mean area under the concentration–time curve from time zero extrapolated to infinity (AUC_{inf}) were observed across the dose range studied (see Supplemental results for further details). The non-linearity was most evident as the dose increased from 30 to 100 mg IV, where dose-normalized AUC_{inf} increased by approximately 100%, indicating a decrease in clearance for this increase in dose. Thereafter, dose-normalized AUC_{inf} only slightly increased as the dose increased from 100 to 3000 mg IV. As expected for an increase in dose, the time to the last measurable concentration of REGN5069 in serum increased from 44 days (30 mg) to 237 days (3000 mg) for the IV doses.

Biomarkers and immunogenicity

Overall, small increases in high-sensitivity C-reactive protein (hsCRP) were observed in individual participants, but these were unrelated to REGN5069 dose, and did not represent a trend indicative of an inflammatory response associated with administration of REGN5069. Among all participants, only one (in the placebo SC group) experienced a significant elevation in hsCRP. This was deemed by the investigator to be unrelated to the study drug and resolved within 5 days. hsCRP levels were generally similar among participants in the total placebo and total REGN5069 groups. No participants were ADA-positive during the study.

Phase 2 study of REGN5069 in patients with knee OA pain

Participants

In total, 259 patients with OA were randomized, of whom 171 received IV REGN5069 (100 mg Q4W, n = 85; 1000 mg Q4W, n = 84) and 88 received matching placebo. Patient disposition is shown in Supplemental Fig. 9. Baseline characteristics are summarized in Supplemental Table 5.

Efficacy of REGN5069

Both doses of REGN5069 failed to meet the primary (change from baseline at week 12 in the WOMAC pain subscale score) and secondary (change in WOMAC total score, physical function and stiffness subscale scores, and PGA scores) efficacy endpoints. There was no statistically significant improvement (numerical decrease) in change from baseline in WOMAC pain subscale score at week 12 for either the REGN5069 100 or the 1000 mg groups compared to placebo (Fig. 6 and Supplemental Table 6).

Safety, tolerability, and immunogenicity of REGN5069

The safety and tolerability of REGN5069 treatment was generally comparable to placebo. A higher percentage of patients experienced ≥ 1 TEAE in the placebo group than in either active treatment group in the treatment period alone, the follow-up period alone, and both the combined treatment and follow-up periods. There were no deaths in the study; eight serious AEs occurred in six patients (Supplemental Tables 7 and 8). No treatment-emergent ADAs were observed for patients who received either 100 or 1000 mg IV REGN5069.

Pharmacokinetics of REGN5069

Mean concentration–time profiles of functional REGN5069 in serum following 100 and 1000 mg IV doses were characterized by an initial distribution phase, followed by a mono-exponential elimination phase for up to 16 weeks for the 100 mg dose and up to 24 weeks for the 1000 mg dose (Supplemental Fig. 10). Thereafter, concentrations in serum decreased more rapidly below ~ 3 mg/L for the 100 mg dose and below ~ 12 mg/L for the 1000 mg dose, consistent with target-mediated clearance of REGN5069. For the 100 mg dose, mean trough concentrations in serum (C_{trough}) at the end of each dosing interval exceeded 3 mg/L (estimated mean C_{trough} for the minimally efficacious dose identified in the mouse CFA inflammatory joint pain model). For the 1000 mg dose at weeks 8 and 12, mean C_{trough} exceeded 80 mg/L (mean C_{trough} for a dose with near-maximal efficacy identified in the mouse artemin-induced thermal hyperalgesia model). See Supplemental results for further details.

Discussion

Results from our preclinical experiments in mice showed that anti-GFRα3 antibodies were efficacious in attenuating allodynic responses in three different chronic joint pain models. In the mouse DMM model of osteoarthritic pain, both REGN1967 and REGN5069 produced significant anti-allodynic effects against tactile allodynia in the ipsilateral limb. Allodynia could be both prevented and reversed in this model, but early treatment did not appear to be disease-modifying for the allodynic

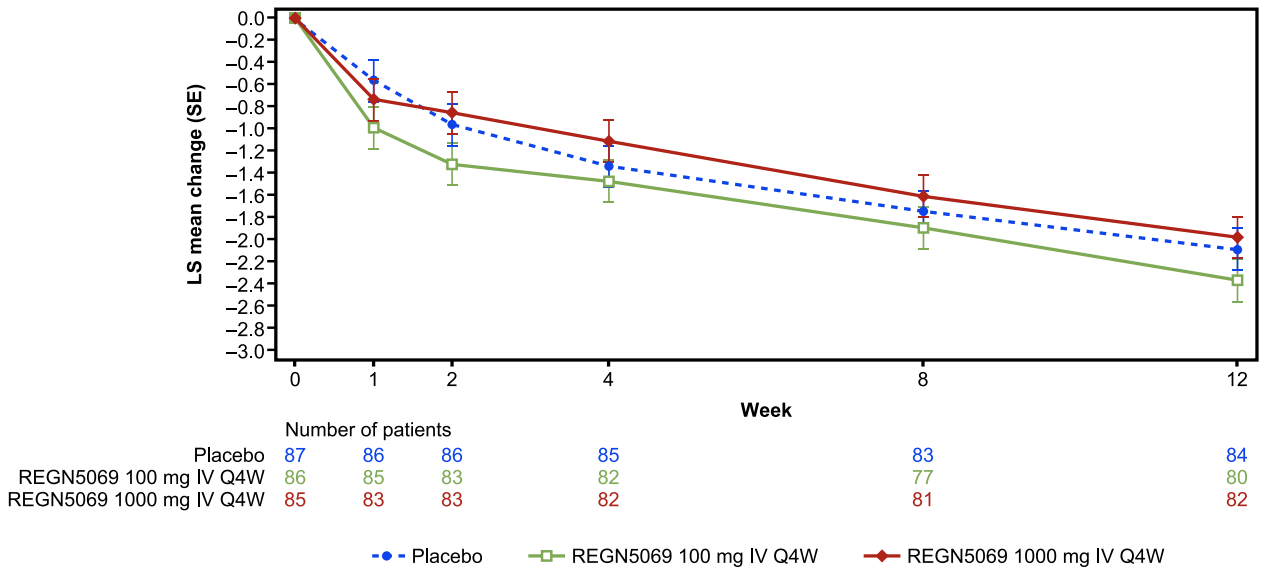

Fig. 6. Least squares mean (± standard error [SE]) change from baseline in WOMAC pain subscale score by visit in the phase 2 study in patients with OA (full analysis set).

state because tactile allodynia developed after antibody withdrawal. In addition, treatment did not appear to be disease-modifying against joint pathology, because μCT scanning of the joints revealed no difference between the treatment groups in osteophyte burden. To increase confidence that GFRα3 plays a role in allodynic responses in mouse models of OA-like joint pain, we tested REGN5069 in a second model of mouse osteoarthritic pain, the MIA model, which models the chondrocyte loss of OA. Consistent with a role in osteoarthritic-like nociception, GFRα3 inhibition prevented tactile allodynia in this second model of OA-like pain, again without discernably impacting pathology (in this case, cartilage). In an effort to distinguish between a role for GFRα3 specifically in osteoarthritic-like joint pain or joint pain in general, we injected animals with intra-knee CFA, a third model of pain in joint and surrounding tissues, and monitored both allodynic and hyperalgesic responses after injection with REGN1967 and REGN5069. CFA induces an inflammatory response in the knee joint and adjacent muscle, which is consistently accompanied by both allodynia and thermal hyperalgesia in the injected joint. Both GFRα3 antibodies were significantly efficacious against tactile allodynia and thermal hyperalgesia in this model; however, neither REGN1967 nor REGN5069 caused a discernible effect on the extent of inflammation induced in this model, suggesting that their primary mode of efficacy was against the nociceptive signal rather than against the inflammatory response.

Given the reproducible anti-allodynic effects of GFRα3 antibodies in three preclinical joint pain models, we tested our lead human antibody to GFRα3 (REGN5069) in a phase 1 'first-in-human' trial and in a phase 2 proof-of-concept trial to treat pain caused by OA in the knee; these studies characterized the PK of REGN5069, and safety appeared similar to placebo, although the phase 2 trial was ultimately terminated early due to lack of efficacy. At higher concentrations in serum, REGN5069 showed a brief distribution (IV administration) or absorption (SC administration) phase, followed by an initial linear elimination phase, in which concentrations declined at a constant rate. At concentrations below approximately 4–10 mg/L, a target-mediated terminal elimination phase followed where concentrations declined more rapidly, suggesting that the antibody reached and engaged target. Unfortunately, we cannot determine whether the antibody sufficiently engaged target in the regions needed for pain relief in our human patients. The higher REGN5069 dose regimen used in the phase 2 trial (1000 mg Q4W) was selected to maintain concentrations of REGN5069 in serum in excess of trough concentrations associated with near-maximal efficacy in the mouse artemin-capsaicin thermal hyperalgesia model and those associated with target saturation in serum, for the duration of each dosing interval. The lower dose regimen (100 mg Q4W) was selected to have adequate separation in exposure from the high dose and achieve efficacy less than that expected for the high dose, but still measurably greater than the control. In contrast to our observations in mice, patients with knee OA pain did not achieve pain relief. Both mouse and human knees contain GFRα3-immunoreactive nerve endings and both the DMM and MIA models are commonly used models for studying OA pain. Additional research will be required to distinguish whether the role or availability of GFRα3 in pain is different in humans than in mice, or whether our preclinical models and/or measures failed to emulate key aspects of human knee OA pain. The difficulty in translating findings from rodent models of OA to human clinical trials has been documented previously and remains a challenge to the development of new treatments (for review see Malfait and Little, 2015).

Of note, our preclinical studies did not directly address the mechanisms by which inhibiting GFRα3 signaling in mice reduces allodynia, but taken together with data from other laboratories (Elitt et al., 2006; Malin et al., 2006; Elitt et al., 2008; Yoshida et al., 2011), it is rational to propose that signaling of the RET/GFRα3 receptor complex sensitizes nociceptive responses in mice, perhaps by sensitizing another nociceptive receptor. GFRα3 and TrpV1 have a 67% co-localization in adult mouse dorsal root ganglia (DRG) neurons, and the GFRα3 ligand artemin has been shown to potently sensitize capsaicin responses in these same neurons *in vitro* (Elitt et al., 2006; Malin et al., 2006). Indeed, artemin increased capsaicin responses significantly more than the pro-nociceptive growth factor NGF in that same experiment. In a separate experiment, the same researchers showed that artemin sensitized both TrpV1 and TrpA1-mediated responses *in vivo* by local administration of either capsaicin or mustard oil (Elitt et al., 2008). Our preclinical data also suggest that exogenously administered artemin does not mediate mouse nociceptive responses via upregulation of NGF, because an NGF neutralizing antibody failed to attenuate artemin-induced thermal hyperalgesia. We also showed that the hyperalgesic effects of NGF in this model do not require GFRα3 signaling, given that anti-GFRα3 antibodies did not prevent NGF-induced hyperalgesia. It appears likely that in this mouse model, the two growth factor pathways induce hyperalgesic responses by converging on similar downstream cellular mechanisms in the DRG nociceptive neurons. Whether similar mechanisms occur in humans, including the NGF-independent nature of the effect through

GFRα3, is unknown.

NGF is released by inflammatory cells and may be released by cells of the bone, especially chondrocytes (Iannone et al., 2002; Pecchi et al., 2014; Jiang et al., 2015), but the potential source of GFRα3 ligand (artemin) release in these models is currently unknown. Artemin expression is highest in rodents during development and is low in adult mouse and human tissues (Honma et al., 2002), although it has also been reported to be expressed in normal adult rodent vascular smooth muscle cells (Honma et al., 2002), especially those found in the dura mater (McIlvried et al., 2010). Artemin has been reported to be expressed in inflamed rodent skin (Ikeda-Miyagawa et al., 2015), which could be relevant in our models given that our outcome measures were all evaluated by evoking pain responses from the plantar surface of the paw. Artemin has been reported to be upregulated in the skin of patients with atopic dermatitis (Murota et al., 2012), who often report substantial pruritus and pain. However, these models involve release of artemin from skin cells, not from cells within the joint. Artemin has not been shown to be constitutively expressed in mouse or human bone or joint tissues, but whether there are local sources of artemin release in the joint during pathological states remains to be determined. In contrast, GFRα3 is expressed in mouse small nerve fibers (Nencini et al., 2018). It is also possible that GFRα3, artemin, or RET are regulated or expressed differently in humans versus mice. Public expression profiling databases suggest that GFRα3 and RET are expressed in both mouse and human DRG, however, RET appears to be more highly expressed in mouse DRG than human (BioGPS, 2022). Whether the high level of RET expression in mouse DRG contributes to any differences in pain responses in mouse versus human is unknown.

The mouse DMM model is considered among the best rodent models of moderate OA because it replicates several features of human OA, including pain, osteophyte development, and cartilage loss (Glasson et al., 2007). However, the finding that REGN5069 did not reduce human OA pain despite showing a benefit in three frequently used models of mouse joint pain (DMM, MIA, and CFA), suggests that some aspects of these models or the measures we used fail to fully replicate the biology or type of pain in human OA. While it is possible that the relative role, location or confirmation of GFRα3 and other pain mediators in rodent versus human could be different and account for some of our findings, our data could suggest failure of other aspects of the preclinical design to capture the type of pain experienced by human OA patients. In our experience, tactile allodynia of the ipsilateral hind paw is the most consistent observable measure of pain-like behavior in the mouse DMM model. We have evaluated other behaviors such as locomotion and weight-bearing in our DMM model and have found either no effect or a small effect size incompatible with evaluating therapeutics. It remains feasible that there are other measures that could be used to better model the pain of human OA. For example, other researchers have shown changes in nocturnal movement, which we did not assess in these experiments (Miller et al., 2012). Because the Von Frey test is used to evoke reflexive allodynic behavior in mouse models of OA, but human OA patients were asked to evaluate un-evoked pain, the nature of the pain measure differs substantially. Malfait and colleagues (Malfait et al., 2013) offer a detailed discussion of evoked versus non-evoked measures in evaluating joint pain in rodents, and it may be important for future work in rodent modelling of the joint pain of OA to identify non-evoked responses that might better translate to human OA pain. In addition, we measured thermal hyperalgesic or mechanical allodynic responses in the ipsilateral paw of mice, while OA patients were asked to evaluate perceptual pain in the knee joint during daily activity and rest. While quantitative sensory testing studies measuring sensitization have shown remote allodynic effects in only a subset of OA patients (Thakur et al., 2014; Sachau et al., 2022) not all OA patients show this effect, and our clinical trial did not specifically address allodynia. Instead, we chose to evaluate OA pain using the WOMAC scale, which measures clinically important and patient-relevant changes including pain and function. Creating or identifying models and endpoints leading to reliable and translatable hyperalgesic responses in rodents more reflective of human OA pain would be an important contribution of future research.

Regardless of the mechanisms of the anti-allodynic effect of inhibiting GFRα3 in mouse models of joint pain, the results observed in human OA patients suggest that treatment with an anti-GFRα3 antibody does not attenuate human OA pain. We have insufficient data to allow us to definitively determine whether the reason for the observed difference in efficacy is due to a translation failure of the models and measures, a difference in the role of GFRα3 in mouse versus human joint pain, or failure of antibody to engage target in regions required for pain relief. Additional work would be needed to understand why GFRα3 antibodies failed to show impact on pain in the clinical setting. Nevertheless, based on the clinical trial results, REGN5069 does not appear to be a promising treatment for human OA pain.

CRediT authorship contribution statement

Selin Somersan-Karakaya: Investigation, Supervision, Data curation, Formal analysis, Writing – original draft, Writing – review & editing. **Kenneth C. Turner:** Conceptualization, Methodology, Data curation, Formal analysis, Writing – original draft, Writing – review & editing. **Luz Cortes-Burgos:** Data curation, Formal analysis, Writing – review & editing. **Jutta Miller:** Conceptualization, Methodology, Investigation, Supervision, Data curation, Formal analysis, Writing – review & editing. **Michael LaCroix-Fralish:** Conceptualization, Methodology, Investigation, Supervision, Data curation, Formal analysis, Writing – original draft, Writing – review & editing. **Veronika Logovinsky:** Conceptualization, Methodology, Investigation, Supervision, Data curation, Formal analysis, Writing – review & editing. **Yamini Patel:** Conceptualization, Methodology, Investigation, Supervision, Data curation, Formal analysis, Writing – review & editing. **Richard Torres:** Conceptualization, Writing – review & editing. **Samit Ganguly:** Data curation, Formal analysis, Writing – review & editing. **Aurora Breazna:** Writing – review & editing. **Michelle DeVeaux:** Data curation, Formal analysis, Writing – review & editing. **Rafia Bhore:** Data curation, Formal analysis, Writing – review & editing. **Min Gao:** Conceptualization, Writing – review & editing, Supervision. **Frank J. Delfino:** Data curation, Formal analysis, Writing – review & editing. **Ashique Rafique:** Data curation, Formal analysis, Writing – review & editing. **Jeanette L. Fairhurst:** Conceptualization, Data curation, Formal analysis, Writing – review & editing. **Charleen Hunt:** Conceptualization, Data curation, Formal analysis, Writing – review & editing. **Robert Babb:** Data curation, Formal analysis, Writing – review & editing. **Ashok Badithe:** Data curation, Formal analysis, Writing – review & editing. **William T. Poueymirou:** Conceptualization, Supervision, Data curation, Formal analysis, Writing – review & editing. **Ronald Surowitz:** Writing – review & editing, Investigation, Supervision. **Sylvie Rottey:** Writing – review & editing, Supervision. **Andrew J. Murphy:** Conceptualization, Writing – review & editing, Investigation, Supervision. **Olivier Harari:** Conceptualization, Writing – review & editing, Investigation, Supervision. **Lynn E. Macdonald:** Conceptualization, Writing – review & editing, Investigation, Supervision. **Susan D. Croll:** Conceptualization, Methodology, Investigation, Supervision, Data curation, Formal analysis, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: All authors apart from Sylvie Rottey are employees or former employees of Regeneron Pharmaceuticals, Inc. Sylvie Rottey is an employee of Ghent University Hospital, Ghent, Belgium, which was contracted by Regeneron Pharmaceuticals, Inc. to conduct the study.

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Data availability statement

Qualified researchers may request access to study documents (including the clinical study report, study protocol with any amendments, blank case report form, and statistical analysis plan) that support the methods and findings reported in this manuscript. Individual anonymized participant data will be considered for sharing once the product and indication has been approved by major health authorities (e.g., FDA, EMA, PMDA, etc.), if there is legal authority to share the data and there is not a reasonable likelihood of participant re-identification. Submit requests to https://vivli.org/.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ynpai.2023.100136.

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