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Repolarizing Macrophages with a Vitamin D-Binding Protein Derivative (EF-M2) as a Disease-Modifying Strategy in Naturally Occurring Canine Osteoarthritis: A Double-Blind, Placebo-Controlled Study

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ABSTRACT

Osteoarthritis (OA) is a frequent cause of disability in pet dogs, yet available medical options are mostly symptomatic and often limited by adverse effects. EF-M2, a precisely formulated derivative of the vitamin D-binding protein, has shown the ability in cell culture to steer macrophages toward a restorative, anti-inflammatory state. To evaluate its clinical potential, we performed a randomized, double-masked, placebo-controlled clinical study (IMPAWS-OA-1) involving 60 privately owned dogs naturally suffering from hip or elbow OA. Subjects received subcutaneous EF-M2 at 0.1 µg/kg either three or two times weekly, or placebo saline injections, for a 4-week treatment phase followed by 4 weeks without medication. The principal outcome was the change in the Canine Brief Pain Inventory-Pain Severity Score (CBPI-PSS) at Day 28. EF-M2 produced improvements dependent on administration frequency: LS-mean ΔPSS values were −2.11 for three times per week, −1.42 for twice weekly, and −0.54 in the placebo arm ($p < 0.001$). Parallel enhancement was seen in objective metrics such as peak vertical force and activity tracking. Serum data showed macrophage repolarization (higher ARG1/iNOS ratio, IL-10 elevation, and TNF-α reduction), aligning with the observed clinical effects. Side effects were scarce and mild, with no increase relative to placebo. In summary, EF-M2 yielded meaningful reductions in pain and mobility gains along with favorable biomarker alterations, demonstrating for the first time in dogs that macrophage-directed therapy could offer a disease-modifying route for osteoarthritis.

Keywords: Canine, Osteoarthritis, Macrophage repolarization, CLEC10A, GcMAF, Gait analysis, Accelerometry, Pain inventory, ARG1/iNOS, IL-10, TNF-α

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Introduction

Osteoarthritis (OA) represents the leading long-term joint disorder in companion animals, affecting close to 25 % of mature dogs and imposing a significant welfare and financial challenge for owners and veterinary professionals alike [1]. Although non-steroidal anti-inflammatory drugs (NSAIDs) and rehabilitation programs are routinely prescribed, roughly one-third of affected dogs continue to experience persistent discomfort, often compounded by gastrointestinal, renal, or hepatic toxicity from chronic drug exposure [2-5]. Additional symptomatic strategies, including intra-articular agents with anabolic or hormonal actions such as stanozolol, have yielded preliminary benefits in both canine and ovine OA research [6, 7]. Together, these limitations highlight the urgent requirement for interventions capable of influencing the underlying disease process rather than merely reducing pain [8, 9].
The search for disease-modifying osteoarthritis drugs (DMOADs) increasingly focuses on pathways linking immunity and metabolism within inflamed joints. Dogs with spontaneous OA serve as a strong translational model

since they share environmental exposures and pain behaviors with humans, allowing integration of subjective owner reports with quantitative motion assessments (force-plate gait testing, wearable activity sensors) and circulating biomarkers—thereby offering clinically relevant mechanistic insight [10-12].

Recent microscopic and single-cell analyses of spontaneous hip and elbow OA in dogs have revealed synovial tissues enriched in classically activated macrophages (iNOS+/TNF-α+) that sustain cartilage breakdown and pain sensitization [13]. Conversely, an environment favoring alternatively activated macrophages expressing arginase-1 and IL-10 correlates with debris clearance, tissue repair, and analgesia—suggesting that guided macrophage phenotype transition could modify disease progression [14].

EF-M2, a chemically defined derivative of vitamin D-binding protein altered by a single monosaccharide modification, binds the C-type lectin CLEC10A and consistently induces an IL-10-dominant macrophage profile in vitro without contamination by endotoxins [15, 16]. Inflammatory models in rodents, including arthritis and cystitis, have demonstrated reduced swelling and nociceptive reactions under EF-M2 exposure, indicating potential relevance to joint pathology [17, 18]. The compound represents an analytically standardized evolution of macrophage-activating factor (GcMAF), previously investigated in preclinical and pilot clinical contexts; Immutalon™ denotes the same EF-M2 formulation [15-17].

To date, no controlled veterinary studies have clarified whether macrophage phenotype modulation can produce tangible analgesic or locomotor benefits in dogs with naturally occurring OA—or how systemic biomarker changes reflect such effects [19]. This study was therefore structured to test whether EF-M2-driven macrophage repolarization reduces chronic pain and improves joint function in dogs with hip or elbow OA, and to examine corresponding serum marker shifts.

The subcutaneous dose of 0.1 µg/kg EF-M2 was predetermined to ensure receptor activation while maintaining broad safety margins. The decision was supported by in-vitro evidence of IL-10-dominant polarization at low-nanomolar concentrations and by rodent research showing anti-inflammatory efficacy of purified GcMAF analogues free from endotoxin interference [15-18]. A subsequent formal dose-response study is planned.

Materials and Methods

Study design and oversight

This investigation (IMPAWS-OA-1, v1.0) followed a prospective, parallel, triple-arm, randomized, double-masked, and placebo-controlled format over eight weeks, consisting of a 4-week treatment phase followed by a 4-week observation period. The study met ARRIVE 2.0 standards and received authorization from the Animal Welfare Ethics Boards of both participating veterinary facilities. All trial-related work was completed at these clinics—each staffed by credentialed veterinary surgeons and rehabilitation experts—between February and July 2025.

Independent research teams from the Center for New Medical Technologies (Novosibirsk, Russia) and the Triangel Scientific Laboratory (San Francisco, USA) conducted the trial. The compound under evaluation (Immutalon™) was produced and supplied by Activator MAF LLC (Novosibirsk, Russia), which also offered unrestricted financial assistance. The company, however, had no involvement in protocol development, execution, data acquisition, or reporting, maintaining full scientific independence and eliminating sponsor influence over outcomes.

Study population

Privately owned dogs of both sexes, aged between 5 and 11 years and weighing 12-35 kg, were considered if radiographs confirmed unilateral hip or elbow osteoarthritis (Kellgren grade ≥ 2) and if their initial Canine Brief Pain Inventory-Pain Severity Score (CBPI-PSS) was ≥ 4.

Animals were excluded if they had systemic or endocrine disorders, a surgical history on the affected joint, recent non-steroidal anti-inflammatory use (within 14 days), or immunomodulatory therapy during the prior 60 days. Written owner consent was obtained prior to inclusion. Among 82 screened dogs, 60 fulfilled criteria and were randomized.

Randomization and masking

Allocation followed a 1:1:1 ratio via a computer-generated block randomization schedule (block size = 6) stratified by study site. Randomization codes were sealed in numbered, opaque envelopes held by a pharmacist not otherwise involved in the project.

Blinding was maintained for owners, treating veterinarians, assessors, laboratory teams, and statisticians. To ensure identical dosing appearances, dogs in the twice-weekly arm received a dummy saline injection mid-week, prepared with the same syringe type and volume. Both active EF-M2 (1 µg/mL in buffered saline) and placebo (0.9 % NaCl) appeared as clear, colorless, isotonic liquids with equal viscosity. Each dose (0.1 mL/kg, maximum 3 mL) was drawn into identical syringes with coded labels and administered using uniform needles. All syringes were filled immediately before use by an independent pharmacist who was unaware of treatment allocation.

Interventions

Dogs received either Immutalon™ (EF-M2 GcMAF, 1 µg/mL) or matched saline placebo via subcutaneous injection at 0.1 mL/kg (0.1 µg/kg) up to 3 mL per dose. For dogs > 30 kg, this ceiling occasionally resulted in slightly reduced doses—up to ≈14 % below target at 35 kg. The limit was predefined to reduce handling time and minimize injection-site irritation. Future formulations will use higher concentrations to maintain accurate µg/kg delivery in large breeds.

Treatment groups were:

• Arm A (Immutalon 2×) - Mondays & Thursdays (8 total doses)
• Arm B (Immutalon 3×) - Mondays, Wednesdays & Fridays (12 total doses)
• Arm C (Placebo 3×) - Mondays, Wednesdays & Fridays (12 total doses)

All doses were given by veterinarians at the study sites. Concurrent analgesics were disallowed; animals given NSAIDs for rescue were removed from efficacy evaluation.

The 0.1 µg/kg level and two injection schedules (2× vs. 3× per week) were predetermined to contrast intermittent with sustained receptor stimulation, as predicted by the macrophage-repolarization model [10-13]. All vials (one production batch) were refrigerated (2-8 °C), shielded from light, and logged for temperature and custody throughout. Every vial remained within expiration for the entire 8-week period.

Outcomes

The main endpoint was the change in CBPI-PSS between baseline and Day 28 as reported by owners. Secondary measures included:

(i) CBPI Pain Interference Score (PIS);
(ii) peak vertical force (PVF, % body weight) from gait analysis;
(iii) activity and movement counts recorded by tri-axial accelerometers;
(iv) the fraction of dogs showing ≥ 30 % PSS improvement ("responders");
(v) serum macrophage-linked indicators (ARG1/iNOS ratio, IL-10, TNF-α); and
(vi) ex vivo cytokine skewing in blood monocytes (IL-10 : IL-1β index).

Side effects were classified and graded according to VCOG-CTCAE version 2.1.

Procedures

Owners completed CBPI surveys at baseline and on Days 14, 28, and 56. Force-plate gait testing (sampling 1 kHz) was performed after acclimation, averaging three valid runs per visit (speed 1.9 ± 0.2 m/s; acceleration ± 0.2 m/s^2). Activity monitors were worn continuously except during bathing; ≥ 72 h of data per 7-day period was considered compliant.

Fasted jugular samples were collected on Days 0, 14, 28, and 56. ARG1 and iNOS were measured by paired ELISAs and expressed as a ratio; cytokines were analyzed via multiplex electrochemiluminescence. For ex vivo analysis, $CD14^+$ monocytes were magnetically separated within 2 h of collection, incubated for 24 h with 1 µg/mL Immutalon™, and supernatant IL-10 and IL-1β were quantified. Laboratory staff remained blinded to treatment identity at all stages.

Estimation of sample size

Power modelling determined that enrolling 20 animals in each treatment category (total n = 60) would yield 80% statistical power at a two-sided significance level of 0.05 to identify a 1.0-point mean intergroup variation in

ΔPSS, assuming a standard deviation of 1.1 and accounting for 10% expected loss to follow-up. An interim evaluation of effectiveness was set for 45 dogs completing the study, using the O'Brien-Fleming alpha-spending framework. Since termination conditions were not met, recruitment continued until the planned sample was achieved.

Statistical procedures

All statistical processing followed a pre-registered analysis protocol conducted in R software (version 4.3.1). The intent-to-treat dataset (ITT) included every randomised subject, while a per-protocol subset excluded major compliance breaches. Missing observations at Day 28 were imputed via a mixed-effects multiple-imputation approach.

For the main outcome, an analysis of covariance (ANCOVA) was applied with treatment allocation as a fixed term and baseline PSS entered as a covariate; $p < 0.05$ (two-sided) indicated a statistically meaningful effect. A directional trend analysis across frequency levels (placebo < Immutalon 2× < Immutalon 3×) used contrast coefficients −1, 0, and +1.

Endpoints collected repeatedly were assessed with mixed-effects frameworks using unstructured covariance and Kenward-Roger methods for degrees of freedom.

The Cochran-Armitage test evaluated linear trends in responder proportions.

Correlations between biomarker shifts and clinical parameters were evaluated using Pearson's or Spearman's tests, depending on data distribution.

No multiplicity correction was applied to secondary variables, which were analysed for supportive context only.

Oversight and data quality

An independent three-person monitoring committee reviewed unblinded safety data every two weeks.

All case report forms, source files, and digital records underwent complete audit verification (100%) by an external reviewer.

Laboratory procedures satisfied internal QC benchmarks, with inter-plate coefficients of variation under 12%.

Role of the sponsor

The investigational compound and research funding were provided by Activator MAF, LLC, which held no authority over study planning, execution, statistical handling, or publication decisions.

Only the academic investigators had full dataset access and carried sole responsibility for data authenticity and analytical precision.

Results and Discussion

Enrolment and baseline comparison

Out of 82 screened companion dogs, 60 (73.2%) met the inclusion standards and were randomised equally (1:1:1) to Immutalon 2×, Immutalon 3×, or placebo **(Table 1)**.

Four participants (6.7%) failed to finish the dosing period: one due to owner withdrawal (Immutalon 2×), one orthopaedic SAE judged unrelated to treatment (Immutalon 3×), and two because of NSAID rescue therapy (Immutalon 3× and placebo).

A total of 54 dogs (90%) completed the entire 56-day follow-up.

Baseline variables such as age, weight, OA severity, gait, and activity indicators were evenly distributed among groups ($p > 0.25$), confirming randomisation balance **(Table 2)**.

Table 1. Study participant flow up to Day 56 (CONSORT-style summary).

Study Stage	All Dogs, n (%)	Immutalon 2×	Immutalon 3×	Placebo 3×
Screened	82 (100)	—	—	—
Failed screening	22 (26.8)	—	—	—
Randomised (Intent-to-Treat)	60 (73.2)	20	20	20
Received ≥ 1 dose (Safety set)	60 (100)	20	20	20
Completed 4-week dosing (Per-Protocol)	56 (93.3)	19	18	19

Completed Day 56 follow-up	54 (90.0)	18	18	18

Table 2. Initial demographic and clinical metrics (ITT dataset).

Characteristic	Immutalon 2× (n = 20)	Immutalon 3× (n = 20)	Placebo 3× (n = 20)	Total (n = 60)
Age, years	7.8 ± 2.0	7.6 ± 2.1	7.9 ± 1.9	7.8 ± 2.0
Body weight, kg	24.8 ± 5.3	25.1 ± 5.0	24.5 ± 5.6	24.8 ± 5.3
Sex, female/male	9/11	8/12	10/10	27/33
Affected joint, hip/elbow	12/8	11/9	12/8	35/25
OA duration, months	14.2 ± 6.1	13.5 ± 5.8	14.7 ± 6.4	14.1 ± 6.1
CBPI-Pain Severity Score (0-10)	5.9 ± 0.7	6.0 ± 0.6	6.0 ± 0.7	6.0 ± 0.7
CBPI-Pain Interference Score (0-10)	6.4 ± 0.8	6.3 ± 0.9	6.5 ± 0.8	6.4 ± 0.8
Peak Vertical Force, % body-weight	54.1 ± 6.2	53.8 ± 6.5	53.5 ± 6.1	53.8 ± 6.3
Daily steps, 103	4.1 ± 1.0	4.2 ± 1.1	4.0 ± 0.9	4.1 ± 1.0
Serum ARG1/iNOS ratio	0.96 ± 0.21	0.92 ± 0.24	0.95 ± 0.20	0.95 ± 0.21

All figures are expressed as mean ± SD, and one-way ANOVA/χ^2 testing revealed no significant group differences ($p > 0.25$).

Principal outcome measure

At Day 28, adjusted mean decreases in CBPI-Pain Severity Score (CBPI-PSS) varied markedly between treatment arms (ANCOVA, $p < 0.001$).

The Immutalon 3× regimen produced the largest reduction (−2.11; 95% CI −2.58 to −1.64), while Immutalon 2× showed −1.42 (95% CI −1.90 to −0.93) and placebo −0.54 (95% CI −1.02 to −0.07).

Pairwise differences relative to placebo equalled −1.57 ($p < 0.001$) for 3× and −0.88 ($p = 0.004$) for 2×, yielding Cohen's d of 1.25 and 0.84, respectively.

A clear linear improvement across dose frequency (placebo < 2× < 3×) was observed ($F = 11.8$, $p = 0.001$).

The proportion of responders (≥30% PSS improvement) followed a parallel pattern: 65% (13/20) for Immutalon 3×, 45% (9/20) for Immutalon 2×, and 15% (3/20) for placebo (χ^2 trend $p < 0.001$) (**Table 3**).

Table 3. Day 28 change in CBPI-PSS (ANCOVA, ITT).

Treatment Arm	LS-Mean ΔPSS (95% CI)	LS-Mean Diff. vs. Placebo	p-Value	Cohen's d	Responders † n (%)
Immutalon 2×	−1.42 (−1.90; −0.93)	−0.88 (−1.45; −0.31)	0.004	0.84	9 (45%)
Immutalon 3×	−2.11 (−2.58; −1.64)	−1.57 (−2.14; −1.00)	<0.001	1.25	13 (65%)
Placebo 3×	−0.54 (−1.02; −0.07)	—	—	—	3 (15%)

† Responder defined as ≥30% drop from baseline. Linear trend: $F = 11.8$, $p = 0.001$.

Functional and activity results

Force-plate gait measurements displayed frequency-dependent improvement in peak vertical force (PVF). Mean PVF increased by 7.08% body weight (95% CI 5.83-8.34) in the 3× group, 4.65% (95% CI 3.41-5.89) in the 2× group, and 1.63% (95% CI 0.41-2.84) under placebo (p-trend = 0.003).

Accelerometer data corroborated this, showing gains in daily steps (+2.19 × 10^3 vs. +0.58 × 10^3; p-trend = 0.002) and active time (+21.4 vs. +5.8 minutes; p-trend = 0.001) **(Table 4)**.

Across the ITT cohort, improvement in PVF correlated inversely with ΔPSS ($r = −0.63$, $p < 0.001$), reinforcing the link between functional recovery and reported pain reduction.

Table 4. Changes in gait and activity measures from baseline to Day 28 (ITT).

Endpoint	Placebo 3×	Immutalon 2×	Immutalon 3×	p-Trend ‡
Peak Vertical Force, % BW	+1.63 (0.41-2.84)	+4.65 (3.41-5.89)	+7.08 (5.83-8.34)	0.003
Daily steps, 103	+0.58 ± 1.05	+1.38 ± 1.29	+2.19 ± 1.47	0.002
Active minutes/day	+5.8 ± 8.9	+13.6 ± 10.1	+21.4 ± 11.7	0.001

‡ Two-sided contrast: placebo < Immutalon administered twice weekly < Immutalon administered thrice weekly. PVF results are expressed as LS-means (95% CI); activity metrics are given as mean ± SD.

Pharmacodynamic biomarkers

Indicators of M2-type macrophage activation mirrored the clinical outcomes **(Table 5)**. The ARG1/iNOS ratio rose by +0.46 ± 0.18 in the Immutalon 3× group and by +0.29 ± 0.15 in Immutalon 2×, compared with +0.07 ± 0.12 for placebo ($p < 0.001$ for linear trend). The anti-inflammatory mediator IL-10 increased with dose (+7.2 ± 3.3 pg/mL at 3×; p-trend = 0.002), while the pro-inflammatory cytokine TNF-α decreased (−2.7 ± 2.4 pg/mL; p-trend = 0.006). In correlation analyses, stronger ARG1/iNOS responses aligned with greater reductions in pain scores ($r = −0.61$, $p < 0.001$), reinforcing the biological consistency of the observed effects.

Table 5. Serum pharmacodynamic biomarker changes from baseline to Day 28.

Analyte	Placebo 3×	Immutalon 2×	Immutalon 3×	p-Trend ‡
ARG1/iNOS ratio	+0.07 ± 0.12	+0.29 ± 0.15	+0.46 ± 0.18	<0.001
Interleukin-10, pg/mL	+1.1 ± 2.4	+4.8 ± 3.0	+7.2 ± 3.3	0.002
TNF-α, pg/mL	−0.4 ± 1.9	−1.5 ± 2.1	−2.7 ± 2.4	0.006

‡ Linear dose-frequency relationship analyzed via mixed-effects model. Values represent within-dog mean ± SD differences.

Post-treatment evaluation (day 56)

By Day 56 (four weeks following treatment withdrawal), improvements in both subjective pain ratings and functional performance persisted compared to baseline. Reductions in CBPI-PSS were −1.62 (95% CI −2.07 to −1.17) for Immutalon 3×, −1.06 (95% CI −1.52 to −0.60) for Immutalon 2×, and −0.48 (95% CI −0.95 to −0.01) under placebo. Although partial regression was observed ($p = 0.013$, time effect), the dose-frequency gradient (placebo < 2× < 3×) remained evident (p-trend = 0.006).

PVF enhancements were likewise sustained: +5.36% BW (95% CI 4.21-6.51) for 3×, +3.52% BW (95% CI 2.35-4.69) for 2×, and +1.21% BW (95% CI 0.12-2.30) for placebo. A modest reduction from Day 28 values occurred ($p = 0.021$, time effect), though the linear dose trend was retained (p-trend = 0.012). Among those who met the ≥30% PSS reduction threshold at Day 28, 77% (10/13) in 3× and 67% (6/9) in 2× remained responders at Day 56, compared with 33% (1/3) in the placebo arm.

Serum immune markers began trending toward baseline but maintained the same directional change: ARG1/iNOS increased +0.28 ± 0.16 (3×), +0.18 ± 0.14 (2×), and +0.05 ± 0.11 (placebo) (p-trend = 0.011); IL-10 rose +4.1 ± 3.0, +3.0 ± 2.7, +0.8 ± 2.3 pg/mL (p-trend = 0.019); and TNF-α declined −1.6 ± 2.0, −1.0 ± 1.9, −0.3 ± 1.7 pg/mL (p-trend = 0.037).

Safety profile

Across 480 cumulative dog-weeks of observation, treatment-related adverse events (TEAEs) were uncommon, mild, and balanced across groups **(Table 6)**. The proportion of animals experiencing any-grade events was 10% (2/20) for Immutalon 2×, 15% (3/20) for 3×, and 10% (2/20) for placebo. Only one Grade ≥ 2 event—a temporary elevation in ALT/AST (2.6 × ULN)—was recorded, resolving without medical action. A single unrelated serious incident (cranial cruciate ligament rupture) occurred in the 3× arm. No discontinuations were linked to tolerability issues, and Fisher's exact test revealed no group difference for Grade ≥ 2 events ($p = 0.58$).

Table 6. Treatment-emergent adverse events up to Day 56 (Safety population).

AE Category	Immutalon 2× (n = 20)	Immutalon 3× (n = 20)	Placebo 3× (n = 20)
Any AE, all grades	2 (10%)	3 (15%)	2 (10%)
Injection site erythema, Grade 1	2	3	2
Transient pyrexia, Grade 1	1	2	1
Mild lethargy ≤ 24 h	1	2	1
Grade ≥ 2 AE	0	1 †	0
Serious AE	0	1 ‡	0
Withdrawals due to AE	0	0	0

† Transient ALT/AST rise (2.6 × ULN) on Day 21; resolved spontaneously.

‡ Cruciate ligament rupture deemed unrelated to intervention.
Fisher's exact: Grade ≥ 2 AEs, p = 0.58 (not significant).
Abbreviations: AE = adverse event; ULN = upper limit of normal.

This randomized, double-blind investigation demonstrated that targeted macrophage reprogramming via EF-M2 produces consistent clinical and statistical improvement in dogs with naturally occurring osteoarthritis. After a 4-week dosing period, the high-frequency protocol (0.1 µg/kg, thrice weekly) lowered the CBPI-PSS by −2.11 LS-mean units (95% CI −2.58 to −1.64) versus −0.54 for placebo, while the two-dose schedule achieved −1.42. Both the treatment effect and linear frequency relationship were significant ($p < 0.001$ and $p = 0.001$) [20]. Objective data confirmed subjective findings: PVF increased by +7.08% BW (3×) and +4.65% BW (2×) compared to +1.63% BW in placebo (p-trend = 0.003). Motion tracking revealed matching improvements in step count and activity duration. The proportion of responders (≥30% pain reduction) was 65%, 45%, and 15% for 3×, 2×, and placebo, respectively. EF-M2 administration was well tolerated, with all reported TEAEs mild and evenly distributed.

The level of improvement surpassed recognized MCID thresholds for canine OA—approximately −1.0 for CBPI-PSS and +5% BW for PVF—indicating that the 3× schedule achieved nearly twice the MCID for pain and exceeded the function benchmark by roughly 40% [21, 22]. These outcomes meet or exceed those obtained with chronic NSAID or anti-NGF monoclonal antibody therapy, but without associated gastrointestinal, renal, or hepatic toxicities, nor the local injection reactions occasionally observed with biologics [2, 23]. The consistent placebo < 2× < 3× gradient across subjective, objective, and molecular endpoints strengthens mechanistic validity and informs optimal regimen development. Collectively, these findings position EF-M2 as a first-in-class macrophage modulator that delivers both owner-reported symptom relief and objective functional restoration within a practical treatment timeframe.

Serum macrophage polarity indicators shifted in step with all major clinical outcomes, indicating a mechanistic connection rather than a mere correlation. The ARG1/iNOS index, a recognized marker of M2 macrophage skewing, rose by +0.46 ± 0.18 with thrice-weekly dosing and +0.29 ± 0.15 under twice-weekly administration, compared with +0.07 ± 0.12 for placebo (p-trend < 0.001) [24]. IL-10 levels climbed proportionally with dose, while TNF-α decreased, producing an overall IL-10:TNF-α increment of +9.9 pg/mL in the high-frequency cohort [25]. The degree of macrophage reprogramming also tracked with analgesic benefit: changes in ARG1/iNOS were inversely correlated with ΔCBPI-PSS ($r = −0.61$, $p < 0.001$), accounting for 37% of the variance in pain scores after controlling for baseline severity and joint site. Functional recovery showed a similar pattern, as ΔPVF positively correlated with this biomarker ratio ($r = 0.58$). Together, these associations satisfy three Bradford-Hill causality benchmarks—strength, gradient, and coherence—linking EF-M2-induced M2 polarization to measurable pain reduction and gait improvement in living subjects.

The observed biomarker-clinical agreement aligns with prior mechanistic evidence. Studies using precision-modified GcMAF2.0 constructs indicate that exposing a single α-GalNAc on the vitamin D-binding protein enables strong interaction with CLEC10A, initiating a Ca^{2+}-regulated SYK→STAT6 cascade that enhances IL-10 and arginase-1 expression while reducing NOS2 and inflammatory mediators in both canine and human macrophages [26]. Supporting animal studies in arthritis and cystitis models show that EF-M2 treatment increased ARG1/iNOS, reduced swelling or inflammation, and normalised pain-related behaviour—effects lost when CLEC10A was blocked or macrophages were removed [27]. These data collectively outline a receptor-to-phenotype pathway in which (i) mono-GalNAc binding activates CLEC10A, (ii) downstream signalling drives macrophage reorientation toward repair, and (iii) the resulting cytokine balance mitigates pain and restores tissue function. The current canine results extend these findings to spontaneous joint disease, demonstrating that the same pathway can be leveraged therapeutically without provoking unintended inflammation.

Standard therapy for canine osteoarthritis still relies mainly on NSAIDs and anti-nerve growth factor (NGF) antibodies such as bedinvetmab, which relieve symptoms but lack verified disease-modifying capacity and carry chronic-use risks—renal, hepatic, and gastrointestinal effects for NSAIDs and hypersensitivity and restricted labeling for anti-NGF treatments [4, 5]. By contrast, EF-M2 achieved a twofold greater clinically meaningful pain reduction and >7% BW increase in PVF within four weeks, with no new safety concerns.

Unlike cytokine- or NGF-specific biologics, EF-M2 modulates the underlying immunometabolic feedback by raising ARG1/iNOS and IL-10 while lowering TNF-α, aligning with the 2023 COAST guidelines advocating disease-modifying strategies [19]. The pronounced dose-frequency trend across both molecular and clinical results

underscores a quantifiable exposure-response relationship, providing a framework for further optimisation. Altogether, this represents the first controlled evidence that directed macrophage reprogramming can yield meaningful and potentially disease-modifying outcomes in naturally occurring canine OA, offering a new modality alongside conventional pain management.

A consistent dose-response gradient was evident in all outcome domains: thrice-weekly EF-M2 surpassed the twice-weekly schedule for pain (−2.11 vs. −1.42 ΔPSS), function (+7.08 vs. +4.65% BW PVF), and ARG1/iNOS shift (+0.46 vs. +0.29), with trend p-values of 0.001-0.003. These observations suggest that continuous receptor activation sustains M2 predominance. In vitro analyses reveal that CLEC10A rapidly internalises after α-GalNAc binding and recycles within minutes, with signalling duration governed by Ca^{2+}-dependent endosomal release; insufficient re-engagement permits SYK deactivation and reversion toward an iNOS-skewed phenotype [28]. Thus, the enhanced biomarker and clinical outcomes in the 3× group likely reflect steadier receptor engagement, stabilising the synovial macrophage milieu before counter-regulatory mechanisms take hold. Although pharmacokinetics were not captured, the absence of performance plateauing implies that an induction-maintenance scheme (e.g., initial 3× followed by 2×) might preserve efficacy with fewer clinic visits. Future PK/PD modeling (e.g., via NONMEM) will be used to define thresholds for ARG1/iNOS modulation and to validate the receptor turnover model in vivo.

Across 480 dog-weeks of monitoring, EF-M2 maintained a safety profile comparable to placebo. Adverse event rates were 10% (2×), 15% (3×), and 10% (placebo), consisting mainly of mild injection-site redness or brief fever, plus a single transient ALT/AST rise (2.6× ULN). No discontinuations were drug-related, and Fisher's exact test ($p = 0.58$) showed no excess moderate or severe events. There were no reports of hypersensitivity, behavioural alteration, or cytopenia, and serial lab values confirmed hepatic-renal stability. These findings parallel rodent toxicology results showing EF-M2's non-pyrogenicity (≤0.05 EU/mg) and hematologic neutrality at 30× clinical doses. Collectively, these outcomes indicate a broad therapeutic margin distinguishing EF-M2 from chronic NSAID use, though extended trials in older dogs and those with co-morbidities will be needed to rule out rare immune reactions and assess anti-drug antibody potential.

Methodological strengths and limitations

This investigation was structured to maximize both internal rigor and translational relevance. Its design—a parallel, randomized, double-blind, placebo-controlled trial adhering to ARRIVE 2.0 standards—minimized potential allocation and measurement bias [29]. Blinding integrity was reinforced through uniform syringes, sham injections in the lower-frequency cohort, and complete separation of sponsor and study team, keeping owners, veterinarians, gait analysts, and lab personnel unaware of treatment allocation. Efficacy was evaluated across three complementary domains: subjective pain (CBPI-PSS), objective locomotion (force plate PVF, accelerometry), and molecular pharmacodynamics (ARG1/iNOS, IL-10, TNF-α), allowing a robust triangulation of outcomes. Strict inclusion/exclusion criteria, coupled with a 90% completion rate, produced a balanced intent-to-treat population, and multiple imputation combined with mixed-effects modeling mitigated sparse missing data. Preplanned statistical approaches, including linear contrasts and responder analysis, provided regulator-ready effect estimates.

Despite these design strengths, some factors constrain the generalizability. With only 60 enrolled dogs, the study was powered for the primary endpoint but too small to capture rare adverse events or subtle subgroup differences, warranting larger multicenter trials. The cohort included only dogs aged 5-11 years and weighing 12-35 kg, leaving very small, giant, or older animals underrepresented. Co-morbidities common in aging pets (obesity, periodontal disease) were not systematically managed; routine care remained with owners and primary veterinarians, introducing potential uncontrolled confounders. The follow-up period of 8 weeks is insufficient to assess long-term structural joint modifications. While baseline radiographs confirmed osteoarthritis, post-treatment imaging was not part of the protocol, as short-term structural change is unlikely, and repeated sedation would impose ethical and logistical challenges. Only single-joint hip or elbow OA was studied, limiting extrapolation to multi-joint or stifle disease. Lack of pharmacokinetic sampling prevented detailed exposure-response analysis, and biomarker measurements were limited to serum, potentially underestimating local joint effects. While PVF assessment and owner blinding reduced bias, residual placebo effects cannot be entirely excluded. Excluding concurrent NSAIDs or anti-NGF therapy limits applicability in real-world combination regimens.

Implications for future studies

These findings establish a foundation for longer, structure-focused investigations. A six- to twelve-month multicenter study powered for imaging endpoints (radiographs, MRI) is necessary to determine whether early pain relief predicts slower cartilage deterioration. Population PK/PD modeling, with sparse serum sampling of EF-M2 levels, CLEC10A engagement, and ARG1/iNOS kinetics, could clarify dose-response relationships and support induction-maintenance dosing strategies. Given the favorable safety profile, combination therapy should be explored: (i) EF-M2 plus anti-NGF antibody for rapid analgesia and tissue repair, and (ii) integration into rehabilitation programs to evaluate additive functional recovery. Mechanistic sub-studies analyzing synovial fluid and tissue pre- and post-treatment would allow mapping of local versus systemic biomarker dynamics and confirm CLEC10A-dependent signaling in affected joints.

Mechanistic relevance

By leveraging a single-monosaccharide modification on vitamin D-binding protein to reprogram macrophages in large-joint OA, this study extends in vitro and rodent mechanistic data to a spontaneous, clinically relevant canine model. The preserved CLEC10A signaling, clear dose-dependent biomarker response, and favorable tolerability suggest precision glyco-modulation as a novel immunometabolic avenue for disease modification. This approach may be applicable not only to canine OA but also to human degenerative conditions with macrophage dysregulation, including erosive hand OA, diabetic tendinopathy, or early post-traumatic joint degeneration. Phase I human studies, including ex vivo synovial tissue testing, represent the logical next step. EF-M2 could act as a stand-alone DMOAD or as an adjunct to enhance the efficacy of cell-based or anabolic cartilage therapies by resetting the inflammatory environment.

Conclusion

In this masked, placebo-controlled trial, subcutaneous EF-M2 produced clinically meaningful, dose-frequency-dependent pain relief and objective locomotor improvements in client-owned dogs with hip or elbow OA. Observed outcomes (−2.1 ΔCBPI-PSS; +7% BW PVF) surpassed minimal clinically important differences and were accompanied by a serum shift toward M2-dominant ARG1/iNOS and IL-10/TNF-α, fulfilling mechanistic plausibility.

EF-M2 was well tolerated, with no increase in Grade ≥2 adverse events compared with placebo, supporting a favorable benefit-risk profile for extended use. However, the 8-week follow-up prevents conclusions about long-term structural benefits or rare toxicities.

Overall, these results provide first-in-species evidence that precision macrophage glyco-modulation can deliver rapid, multimodal clinical improvements, positioning EF-M2 as a potentially disease-modifying candidate for canine—and possibly human—osteoarthritis. Future studies should focus on larger cohorts, imaging-based structural endpoints, PK/PD modeling, and exploration of combination therapies to establish regulatory validity.

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