RMD Open
Rheumatic & Musculoskeletal Diseases

ORIGINAL RESEARCH

Oral enzyme combination therapy reduces systemic inflammation, urinary CTXII and pain in knee osteoarthritis: a proof-of-mechanism, randomised, crossover, double-blind, placebo-controlled trial

Yves Henrotin ,[1] Thomas Pap ,[2] Siddhartha Lieten,[3] Valérie Badot,[4] Jean-Emile Dubuc,[5] Didier Urbin-Choffray,[6] Maximilian von Eynatten ,[7] Odd Erik Johansen ,[7] Stefanie Rau ,[7] Karl Brabants[8,9]

To cite: Henrotin Y, Pap T, Lieten S, *et al*. Oral enzyme combination therapy reduces systemic inflammation, urinary CTXII and pain in knee osteoarthritis: a proof-of-mechanism, randomised, crossover, double-blind, placebo-controlled trial. *RMD Open* 2025;**11**:e005433. doi:10.1136/rmdopen-2025-005433

► Additional supplemental material is published online only. To view, please visit the journal online (https://doi.org/10.1136/rmdopen-2025-005433).

YH and TP contributed equally.

Preliminary results were presented as posters at the 2024 Osteoarthritis Research Society International (OARSI; doi: 10.1016/j.joca.2024.02.866), 2024 European Alliance of Associations for Rheumatology (EULAR; doi: 10.1136/annrheumdis-2024-eular.3713) and 2024 French Society of Rheumatology (SFR) congresses.

Received 15 January 2025
Accepted 30 July 2025

For numbered affiliations see end of article.

Correspondence to
Professor Yves Henrotin; yhenrotin@ulg.ac.be

ABSTRACT

Objectives Oral enzyme combination (OEC) therapy with bromelain, trypsin and rutoside reduces pain and improves function in patients with knee osteoarthritis (OA). Here, we investigated several potential biological mechanisms underlying the clinical effects of OEC therapy in patients with established knee OA with respect to innate immunity, systemic inflammation and cartilage turnover (EudraCT 2020-003154-80, NCT05038410).

Methods Patients (age ≥40 years, body mass index (BMI) ≤35 kg/m^2) with symptomatic knee OA were randomised to either placebo or OEC, administered 2×3 tablets/day, for 8 weeks before crossing over after a 4-week washout period. Different markers exploring innate immunity, inflammation and cartilage matrix degradation have been measured in the blood using immunoassays or cytometric methods. Data from the modified intention-to-treat population (mITT) were analysed using a generalised linear mixed model. No correction for multiple comparisons was made due to the exploratory nature of the study.

Results Altogether, 45 patients were randomised; 43 completed both treatment sequences (mITT; mean age: 63.3 years; mean BMI: 27.4 kg/m^2; mean global Knee injury and Osteoarthritis Outcome Score (KOOS): 48.7). OEC significantly increased levels of α2-macroglobulin (p=0.038) and interleukin-10 (p<0.0001) while decreasing urinary carboxyl-terminal cross-linked telopeptide of type II collagen (p=0.038). Patients administered OEC exhibited significant improvements in KOOS Pain (p=0.0464) and Symptoms (p=0.026) subdomains but not globally. OEC was well tolerated, with no serious related adverse events reported in either group.

Conclusions One of the key findings of this proof-of-mechanism study is that OEC modulates IL-10 production, suggesting an anti-inflammatory effect in patients with knee OA. This main finding contributes to explaining the effects of OEC on pain and function in these patients.

Trial registration number NCT05038410.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Currently, no disease-modifying treatment for knee osteoarthritis (OA) is available. Oral enzyme combination (OEC) has demonstrated efficacy in relieving pain in knee OA and other conditions.

WHAT THIS STUDY ADDS

⇒ Here, we show that in patients with knee OA, OEC increased serum α2-macroglobulin, the anti-inflammatory cytokine IL-10 and decreased urinary CTX-II levels while improving patient algo-functional status.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ OEC has the potential to be a safe disease-modifying oral treatment for knee OA. Its use in clinical practice could facilitate physical activity, a core treatment of knee OA, and limit the consumption of non-steroidal anti-inflammatory drugs and analgesics.

INTRODUCTION

Osteoarthritis (OA), a chronic condition affecting the joint and its tissues, is clinically defined by pain, stiffness, deformity, reduced range of motion and muscle atrophy and dysfunction.[1–3] In OA, joint tissues undergo a progressive loss of cartilage, bone remodelling and synovium inflammation.[1 2] A systematic analysis for the Global Burden of Disease Study 2021 for OA found that in 2020, the knee was the most common site of OA with a global age-standardised prevalence of over 4300 cases per 100 000.[4] Patients with knee OA experience substantial disability and impaired quality of life, and OA-associated

physical inactivity due to pain increases the risk of all-cause mortality, cardiovascular disease, osteoporosis, as well as depression and anxiety[5 16].

Current knee OA treatments aim to reduce pain and improve joint function. The Osteoarthritis Research Society International, American College of Rheumatology, American Academy of Orthopaedic Surgeons and European Alliance of Associations for Rheumatology guidelines include both non-pharmacological (exercise, education and weight reduction) and pharmacological (topical or oral non-steroidal anti-inflammatory drugs (NSAIDs) and intra-articular corticosteroids) approaches[7 8–11]. While pharmacological treatments improve symptoms, their clinical efficacy is weak to moderate, and their long-term use, particularly that of oral NSAIDs, may induce adverse effects.[8–10 12 13] Consequently, guidelines recommend that the consumption of oral NSAIDs be limited to the lowest dose and shortest time possible.[8–10] As such, effective but safer alternative pharmacological options would benefit patients with OA.

Oral enzyme combination (OEC) therapy with bromelain, trypsin and the flavonoid rutoside has been reported to significantly improve pain, joint stiffness, functional disability and mobility in several painful musculoskeletal disorders, including knee OA.[13] In clinical trials where OEC or diclofenac was administered to patients with knee OA, OEC was not less effective than diclofenac in reducing pain and/or improving knee function.[14–18] This finding was recapitulated in a pooled reanalysis of individual patient-level data from six clinical trials, which further indicated that the safety profile of OEC was superior to that of diclofenac.[19] The exact pharmacokinetics and pharmacodynamics of OEC and the mechanisms by which it exerts its clinical effects remain poorly understood, although previous studies have explored some potential mechanisms of action. For example, trypsin, a serine protease and bromelain, a cysteine protease, are both thought to modulate inflammatory and immune responses by interacting with the acute-phase protein α2-macroglobulin (A2M) to enable subsequent binding of pro-inflammatory cytokines.[20–22] The flavonoid rutoside has been suggested to have antioxidant and anti-inflammatory effects, which has led to its use in combination with proteolytic enzymes.[23] In preclinical OA models, rutoside reduced cartilage degradation scores and protected articular chondrocytes against hydrogen peroxide-induced oxidative stress.[24 25] Regardless, a more complete understanding of the systemic biological mechanisms of action of OECs remains incomplete. Therefore, this clinical trial was designed to evaluate specifically, in patients with established knee OA (1) as main goal the biological effects of OEC on innate immunity, systemic inflammation and cartilage turnover and (2) as secondary outcomes, potential clinical effects.

METHODS

Design

This study was a randomised, double-blind, placebo-controlled, single crossover, multicentre, exploratory clinical trial (WOBE-SMART, EudraCT 2020-003154-80 and NCT05038410) involving individuals with symptomatic knee OA (figure 1). Patients were recruited from five sites across Belgium from April 2021 to October 2022.

Eligible patients were centrally randomised at baseline to an intervention sequence in a 1:1 ratio using a pre-established randomisation list with block sizes of two or four. Randomisation was stratified by site. Study participants who withdrew during the active recruitment period were replaced.

After the first 8 weeks of treatment with either placebo or OEC, patients had a 4-week washout period before crossing over to their respective alternative arm of OEC or placebo for an additional 8 weeks. Primary outcome

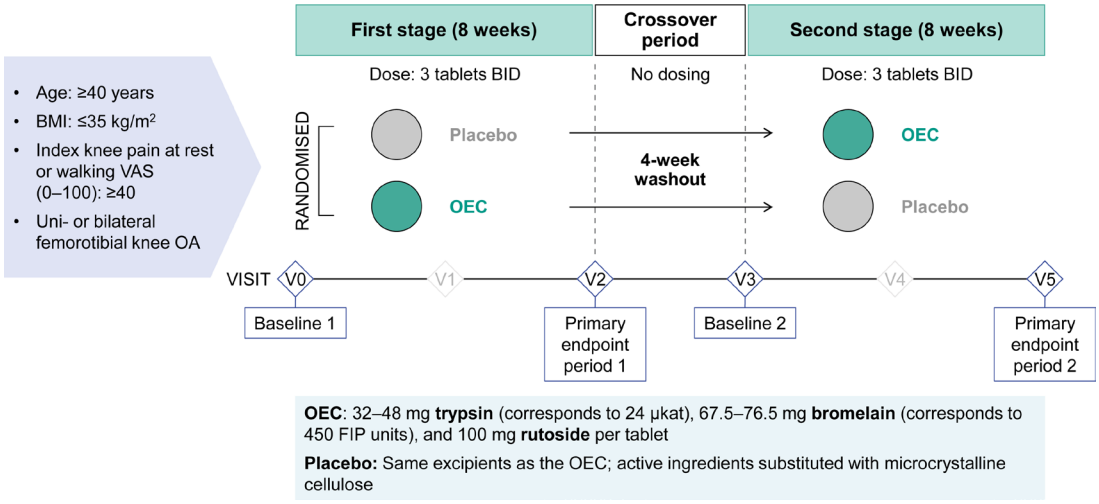

Figure 1 Trial design. This study was a randomised, double-blind, placebo-controlled, single crossover, multicentre, exploratory clinical trial. Treatment duration was 8 weeks with a 4-week washout period. BID, bis in die; BMI, body mass index; FIP, Fédération Internationale Pharmaceutique; OA, osteoarthritis; OEC, oral enzyme combination; V, visit; VAS, Visual Analogue Scale.

objectives were assessed following 8 weeks of intervention with placebo or OEC. Following the completion of the core 20-week study period, an additional 16-week extension was initiated—the results from this extension will be reported elsewhere.

Patients

Individuals were eligible if they were aged ≥40 years, had a body mass index ≤35 kg/m^2, had pain on the index knee at rest or walking (≥40 on a 0–100 Visual Analogue Scale (VAS)), and had symptomatic OA of the knee for more than 6 months (radiological Kellgren-Lawrence grade II–III). Key exclusion criteria included knee prosthesis, recent trauma or arthroscopy, and certain pharmacotherapies or diet (oral prednisolone, local corticosteroid injections, recent use of symptomatic slow-acting drugs for OA or dietary supplements). For full inclusion and exclusion criteria, please see the online supplemental file.

Interventions

The active investigational product was Wobenzym, an OEC containing bromelain (67.5–76.5 mg), trypsin (32–48 mg) and rutoside trihydrate (100 mg) (This formulation is approved as a drug under the name 'Wobenzym' in Germany, used in this trial, as well as under the name of 'Phlogenzym' in Czech Republic and some other Eastern European countries). To ensure blinding of the study medication, OEC and its matching placebo were provided from the same batch number and in identical tablet dosing forms. More specifically, the matching placebo was identical in form and shape and used the same enteric coating as the OEC, but the active ingredients were replaced with microcrystalline cellulose. Both treatments were dosed as three tablets twice a day, in the morning and the evening, taken at least 30 min before or 90 min after a meal.

Outcomes

The primary outcome objectives were to explore the effect of OEC treatment on (1) the innate immune response, (2) markers of systemic inflammation and (3) markers of cartilage turnover. Markers of innate immune response included A2M, macrophage 1/macrophage 2 (M1/M2) polarisation and nucleotide-binding domain, leucine-rich repeat containing family, pyrin domain-containing-3 (NLRP3) inflammasome. Systemic inflammatory markers included interleukin-10 (IL-10), interleukin-1β (IL-1β), interleukin-4 (IL-4), interleukin-6 (IL-6) and tumour necrosis factor alpha (TNFα). Cartilage biomarkers included urinary carboxyl-terminal cross-linked telopeptide of type II collagen (uCTXII), serum Coll2-1 (s-Coll2-1), serum Coll2-1NO2 (s-Coll2-1NO2), serum cartilage oligomeric matrix protein and serum N-propeptide of collagen IIA. Further details on assay techniques, sampling, processing and storage conditions can be found in the online supplemental file.

Secondary outcome objectives included the serum concentrations of transforming growth factors (TGF-β1, TGF-β2, TGF-β3) and the Knee injury and Osteoarthritis Outcome Score (KOOS). The KOOS is a patient-reported outcome measure used in knee injury and/or OA that has a global score as well as five subdomain scores: Pain, Symptoms, Activities of Daily Living, Sport and Recreation Function and Knee-Related Quality of Life.[26 27] Each subdomain is scored from 0 (extreme knee problems) to 100 (no knee problems).[26] Other secondary outcome objectives included patient-reported knee pain at rest and while walking as measured on a VAS of 0–100 with 0 representing 'no pain' and 100 representing 'pain as bad as it could possibly be'; use of rescue medication and cumulative analgesic consumption scores[28]; and clinical chemistry measurements using immunoassays, which measured the concentration of myokines (eg, myostatin), haemoglobin, haematocrit, erythrocyte count, triglyceride (TG), high-density lipoprotein, glucose, high-sensitivity C reactive protein (hsCRP), uric acid, coagulation markers and key hepatic enzymes. Post hoc exploratory analyses were performed for hsCRP and interleukin-1 receptor antagonist (IL-1RA). Safety outcomes included discontinuation rates and adverse events (AEs) as coded using the Medical Dictionary for Regulatory Activities.

Statistical analyses

This clinical study was explorative in nature; thus, sample sizes were not formally calculated. However, to inform the study size, we applied an approximate calculation based on an expected difference of 10% between OEC and placebo in the collagen biomarker Coll2-1NO2 and a type I error (alpha) of 5%, assuming a 15% drop-out. The expected difference of 10% was selected as an estimate of clinically relevant changes. Coll2-1NO2 was chosen as a surrogate marker because of its association with collagen degradation and local inflammatory processes, its correlation with disease severity and progression, and its robustness to environmental and experimental conditions.[29 30] To ensure a power of 80% to 90%, we targeted 20 randomised patients per treatment arm.

The modified intention-to-treat (mITT) population included all randomised patients with at least one post baseline assessment for any primary efficacy endpoint. The patients were included in the group they were randomised to irrespective of the actual study intervention received. Data for the mITT population were analysed using a generalised linear mixed model (GLMM) after 8 weeks of treatment based on the changes from the baseline of each period before and after crossover. The GLMM accounted for the presence of repeated measures as well as the crossover design, and no multiplicity adjustments were implemented. The GLMM included fixed effects for treatment, period and sequence, with subject as a random effect. Baseline values were included as covariates when relevant.

An unstructured covariance matrix was used to model within-subject correlations, consistent with best practices for crossover designs.

Mean changes between baseline and the end of the treatment period for the primary outcome assessments are provided for both treatment periods for OEC and placebo. The means are based on the combination of the identical treatment arms during the crossover periods. Accordingly, each treatment period was compared with its specific baseline (ie, data from V0 (visit 0) served as baseline for the first treatment period, while data from V3 (visit 3) served as baseline for the second treatment period). Treatment effects are given as the ratio of the geometric mean (G_{mean}) of the percent change from baseline of OEC over placebo (G_{mean} OEC/G_{mean} placebo) with a 95% CI.

The safety population included all patients who received one or more dose of OEC. Statistical analyses were performed using SAS software (V.9.4, SAS Institute) without replacement of missing data. Subjects with at least one post baseline value were included in the modified ITT (mITT) analysis. No imputation was done; missing data were handled via mixed models (GLMM/GEE) on observed data only. Nominal p values <0.05 were defined as statistically significant, and no correction for multiple comparisons was made.

Patient and public involvement

Patients and/or the public were not involved in the design, conduct, reporting or dissemination plans of this research.

RESULTS

Patients

Between April 2021 (first participant signed informed consent) and October 2022 (trial end), 45 individuals were randomised, and 43 completed both treatment sequences (mITT population). The five investigational sites recruited between 5 and 14 patients each. Of these 43 patients, 21 patients started with a placebo and thereafter crossed over to OEC (Placebo/OEC group), whereas 22 patients were initially on OEC and thereafter received a placebo (OEC/Placebo group). An overview of patient disposition is depicted in figure 2. Overall, major protocol deviations were infrequent, and a detailed summary is presented in the online supplemental file.

Baseline demographics and characteristics were generally similar across both treatment groups, except that VAS knee pain intensity at walking was significantly higher in patients assigned to OEC/placebo than in patients assigned to placebo/OEC (table 1). Additionally, OEC/placebo patients had lower KOOS global scores than

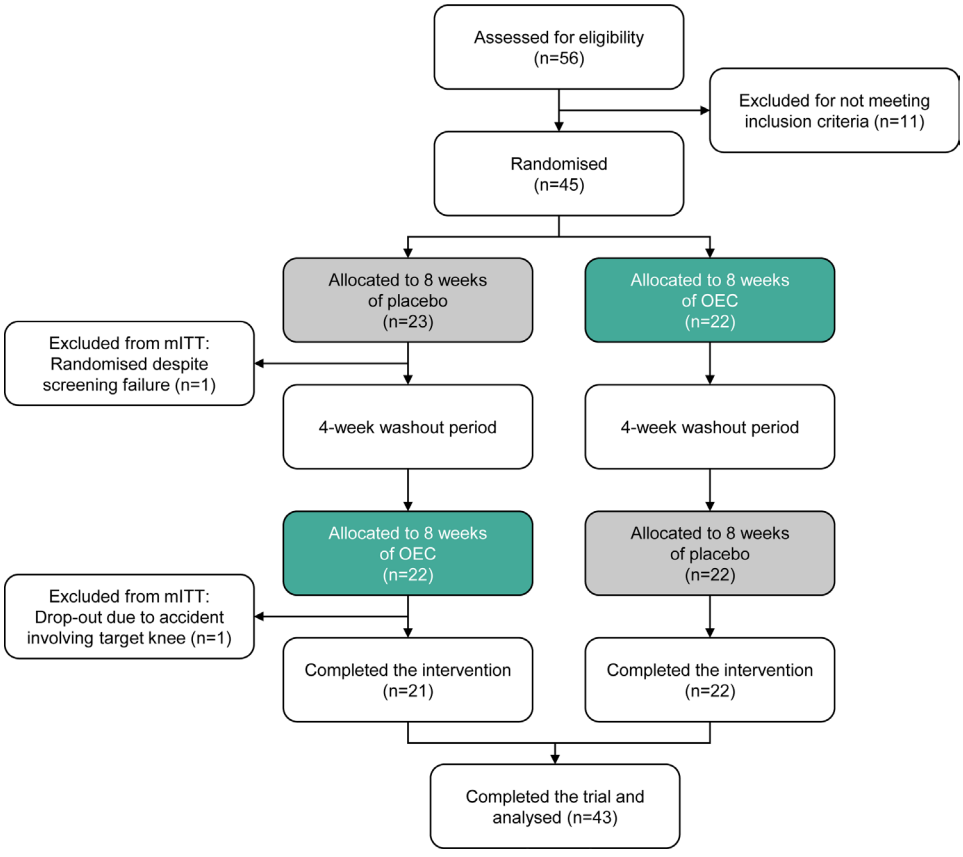

Figure 2 Patient disposition. Numbers represent the mITT population. mITT, modified intention-to-treat; OEC, oral enzyme combination.

Table 1 Baseline demographics and characteristics by intervention sequence

Characteristic	All (N=43)	Placebo/OEC (n=21)	OEC/Placebo (n=22)
Age, years, mean (SD)	63.3 (10.4)	64.1 (12.2)	62.5 (8.5)
Sex, n (%)			
Male	19 (44.2)	11 (52.4)	8 (36.4)
Female	24 (55.8)	10 (47.6)	14 (63.6)
Body mass index, kg/m^2, mean (SD)	27.4 (3.5)	27.6 (3.3)	27.3 (3.7)
Blood pressure, mm Hg, mean (SD)			
Systolic	128.4 (19.7)	129.1 (20.3)	127.8 (19.6)
Diastolic	80.1 (10.9)	78.2 (10.9)	81.9 (10.8)
Radiological Kellgren-Lawrence grade II or III, n (%)	43 (100)	21 (100)	22 (100)
VAS knee pain intensity, mm, mean (SD)			
At rest	38.3 (21.7)	39.2 (16.7)	37.4 (25.9)
At walking	56.9 (13.3)	51.0 (11.2)	62.5 (12.9)
KOOS global score, %, mean (SD)	48.7 (12.9)*	52.6 (12.1)†	45.0 (12.9)‡

Patients assigned to OEC/placebo had significantly higher baseline VAS knee pain intensity at walking compared to patients assigned to placebo/OEC. OEC/placebo patients also had lower KOOS global scores than placebo/OEC patients, although this difference was not statistically significant.

*n=39.
†n=19.
‡n=20.
KOOS, Knee injury and Osteoarthritis Outcome Score; NA, not applicable; OEC, oral enzyme combination; VAS, Visual Analogue Scale.

placebo/OEC patients, although this difference did not reach statistical significance.

Across all patients, the mean (SD) age was 63.3 (10.4) years; most patients were female (n=24, 55.8%). All patients had a Kellgren-Lawrence grade of II or III, and their mean (SD) KOOS global score was 48.7 (12.9).

Primary outcome objectives

Markers of innate immune response

After the 8-week treatment period, baseline mean A2M increased from 1.60 (SD 0.37) to 1.62 (SD 0.36) g/L in the OEC group and decreased from 1.68 (SD 0.35) to 1.66 (SD 0.37) g/L in the placebo group. The observed total +3% between-group difference in change of A2M from baseline was statistically significant and in favour of OEC (G_{mean} OEC/G_{mean} placebo: 103%; 95% CI: 100% to 105%; p=0.038; figure 3A,B). No significant between-group differences were found for other innate immunity markers (p>0.05; online supplemental table S1).

Markers of systemic inflammation

After the 8-week treatment period, baseline mean IL-10 increased from 208.5 (SD 0.57) to 302.3 fg/mL (SD 0.83) in the OEC group and from 208.0 (SD 0.53) to 213.6 (SD 0.45) fg/mL in the placebo group. The observed total +41% between-group difference in change of IL-10 from baseline was statistically significant and in favour of OEC (G_{mean} OEC/G_{mean} placebo: 141%; 95% CI: 122% to 162%; p<0.0001; figure 3C,D). No significant between-group differences were found for other markers of systemic inflammation (p>0.05; online supplemental tables S2 and S3).

Markers of cartilage turnover

After the 8-week treatment period, baseline mean uCTXII decreased from 493.2 (SD 0.63) to 488.3 (SD 0.83) ng/mL in the OEC group but increased from 485.4 (SD 0.79) to 556.6 (SD 0.70) ng/mL in the placebo group. The observed total −11% between-group difference in change of uCTXII from baseline was statistically significant and in favour of OEC (G_{mean} OEC/G_{mean} placebo: 89%; 95% CI: 80% to 99%; p=0.0381; figure 3E,F). No significant between-group differences were found for other serum collagen markers (p>0.05; online supplemental tables S4 and S5).

Secondary outcome objectives

Transforming growth factor-β

OEC treatment after 8 weeks increased TGF-β1 and TGF-β3 levels compared with placebo, although only the increase in TGF-β3 was significant (increase in the OEC group from baseline 26.23 (SD 0.48) to 30.74 (SD 0.66) ng/mL and in the placebo group a decrease from 27.32 (SD 0.42) to 26.17 (SD 0.44) ng/mL; +100% between-group difference, Gmean OEC/Gmean placebo: 200%; 95% CI: 165% to 244%; p=0.0012; figure 4; online supplemental tables S6 and S7). By contrast, TGF-β2 levels were decreased following OEC and placebo treatment, with the magnitude of the decrease significantly smaller in patients treated with OEC (p=0.0273).

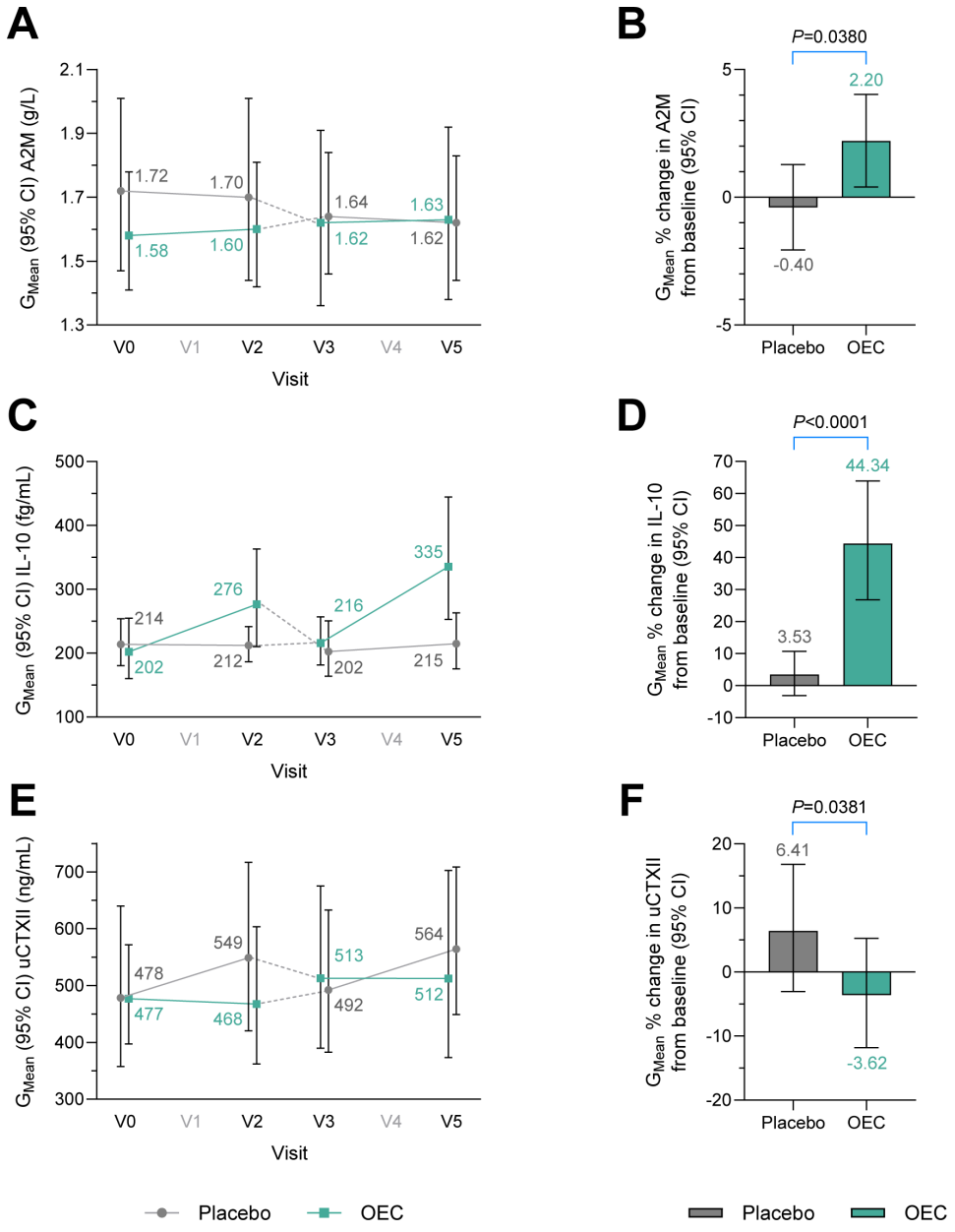

Figure 3 Mean absolute changes during V0–V5 and mean per cent change from baseline in A2M (A, B), IL-10 (C, D) and uCTXII (E, F) in patients treated with OEC and placebo. A2M, α2-macroglobulin; G_{mean}, geometric mean; IL-10, interleukin-10; OEC, oral enzyme combination; uCTXII, urinary carboxyl-terminal cross-linked telopeptide of type II collagen; V, visit.

Knee injury and Osteoarthritis Outcome Score

After 8 weeks of treatment, scores in the KOOS Pain (increase in the OEC group from baseline 54.4 (SD 14.95) to 58.9 (SD 17.62)% and in the placebo group from 56.1 (SD 14.41) to 56.2 (SD 19.18)%; +3% between-group difference, Change in response mean OEC/Gmean placebo: 103%; 95% CI: 100% to 107%; p=0.0464) and Symptoms (increase in the OEC group from baseline 60.4 (SD 13.94) to 65.1 (SD 16.36)% and in the placebo group a decrease from 64.6 (SD 12.85) to 64.4 (SD 18.18)%; +4% between-group difference, Change in response mean OEC/Gmean placebo: 104%; 95% CI: 100% to 107%; p=0.0257) subdomains were significantly increased in the OEC group relative to placebo (figure 5), indicating improvements. No significant between-group differences were observed in the global score or other KOOS subdomains (p>0.05; online supplemental tables S8 and S9).

Patient-reported knee pain as measured by VAS

Changes in patient-reported knee pain from baseline to 8 weeks of treatment, both at rest and while walking, were comparable between the OEC and placebo groups (p>0.05; online supplemental tables S10 and S11).

Rescue medication

Compared with placebo, fewer individuals in the OEC group used rescue medication for general and/or knee pain, although this difference was not statistically validated (online supplemental table S12). Cumulative

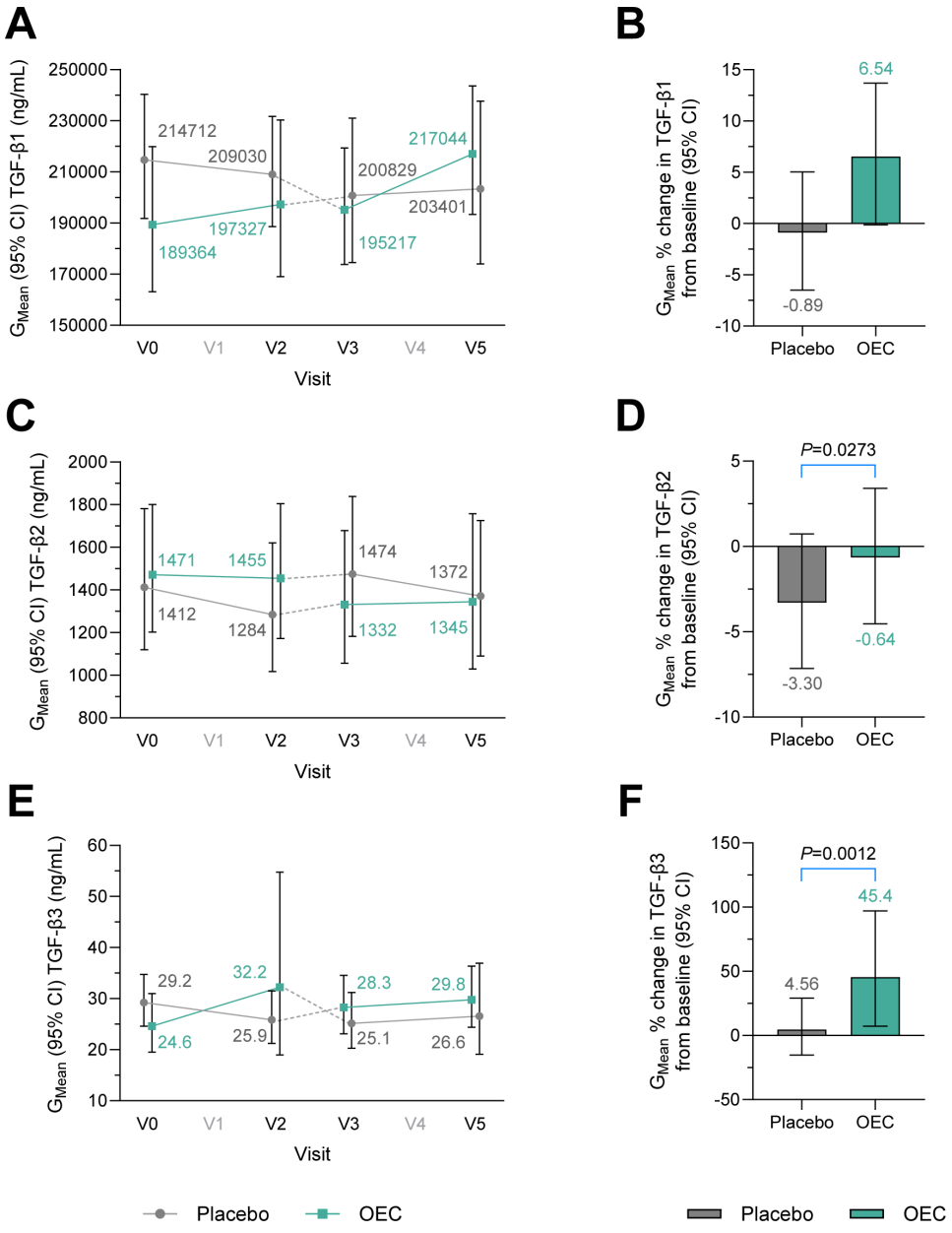

Figure 4 Mean absolute changes during V0–V5 and mean percent change from baseline in TGF-β1 (A, B), TGF-β2 (C, D), and TGF-β3 (E, F) in patients treated with OEC and placebo. G_{mean}, geometric mean; OEC, oral enzyme combination; TGF, transforming growth factor; V, visit.

analgesic consumption scores, which integrate qualitative (potency) and quantitative (dose measurement) information to evaluate analgesic consumption,[28] did not differ between placebo and OEC-treated patients (p>0.05; online supplemental table S13).

Post hoc exploratory analyses

To further explore the effect of OEC treatment on the anti-inflammatory cytokine IL-10, we performed additional subgroup analyses by grouping patients according to baseline (V0) hsCRP tertiles (low, medium and high baseline hsCRP). Systemic IL-10 levels were significantly increased in patients treated with OEC whose baseline hsCRP levels were >0.9 mg/L but not in those with low baseline hsCRP (online supplemental table S14 and 15). Moreover, the ratio of a pro-inflammatory to anti-inflammatory systemic milieu (ie, IL-10/hsCRP) was significantly increased after OEC treatment vs placebo, suggesting a potential anti-inflammatory shift. Additional details of the exploratory analyses are available in the online supplemental file.

Safety and tolerability

Overall, treatment with OEC for 8 weeks was well tolerated, with no serious related AEs reported in either group. The number of patients experiencing at least one treatment-emergent AE (TEAE) was comparable between OEC (29/45) and placebo (38/45). TEAEs related to the investigational product were reported in 8 and 4 patients in the OEC and placebo groups, respectively (table 2).

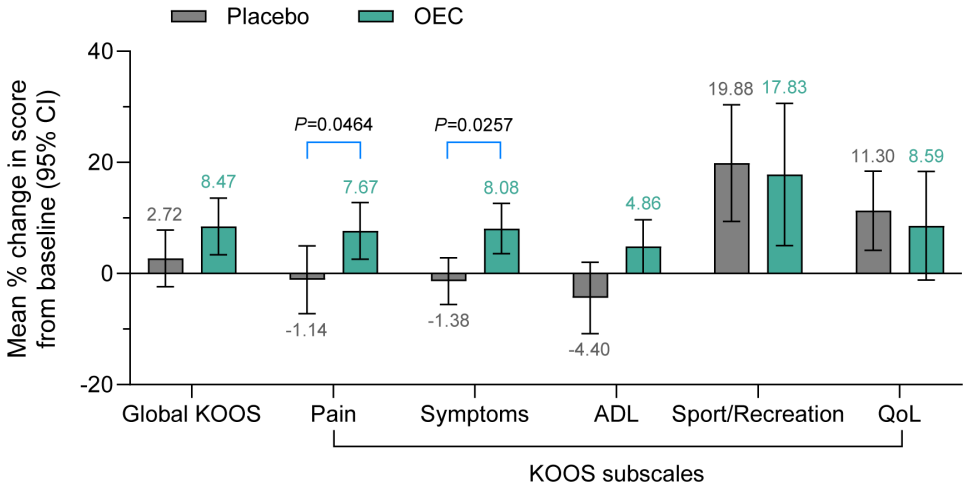

Figure 5 Mean per cent change from baseline in KOOS global and subdomain scores in patients treated with OEC and placebo. ADL, activities of daily living; KOOS, Knee injury and Osteoarthritis Outcome Score; OEC, oral enzyme combination; QoL, quality of life.

The most frequent TEAE related to the investigational product in the OEC group was abdominal pain upper (table 3).

DISCUSSION

This study explored the biological effects of OEC on markers of innate immunity, systemic inflammation and cartilage turnover, thereby fostering our understanding of the clinical effects of OEC in OA. Compared with placebo, OEC significantly elevated levels of A2M with a difference of 3% and the anti-inflammatory cytokine IL-10 with a difference of 41%, and significantly reduced levels of the urinary cartilage biomarker CTXII with a difference of −11%. Furthermore, OEC significantly increased TGF-β3 levels relative to placebo (100% difference), and while TGF-β2 levels were reduced in both OEC and placebo, the magnitude of reduction was significantly smaller in OEC (7% difference). Clinically, patients treated with OEC experienced greater improvements in the KOOS Symptoms (4% difference) and Pain (3% difference) subdomains. These differences persisted despite both treatment groups having used comparable amounts of rescue analgesia and reporting similar pain levels as measured by VAS, although it should be noted that baseline VAS knee pain intensity at walking was significantly higher in patients assigned to OEC/placebo than patients assigned to placebo/OEC. Overall, OEC was well tolerated with a comparable safety and tolerability profile to placebo.

A2M is a protease inhibitor and acts as an acute-phase protein.[21 22] Here, we observed that OEC increased serum A2M levels—these results are consistent with previous

Table 2 Summary of overall TEAEs in the safety population (N=45)

	Placebo (N=45)	OEC (N=45)
Patients with at least one AE, n (%)		
TEAE	38 (84.4)	29 (64.4)
Mild TEAE	27 (60.0)	24 (53.3)
Moderate TEAE	18 (40.0)	14 (31.1)
Severe TEAE	3 (6.7)	1 (2.2)
TESAE	1 (2.2)	0 (0)
TEAE related to investigational product*	4 (8.9)	8 (17.8)

*TEAE related to investigational product is defined as an AE that is 'probably related' or 'related' to the treatment.
AE, adverse event; OEC, oral enzyme combination; TEAE, treatment-emergent AE; TESAE, treatment-emergent serious AE.

Table 3 TEAEs related to investigational product in the safety population by Preferred Term (N=45)

	Placebo	OEC
Total number of TEAEs related to investigational product	7	13
Abdominal distension	1	1
Abdominal pain upper	1	3
Arthralgia	0	1
Diarrhoea	3	1
Dyspepsia	1	0
Flatulence	1	1
Gastrointestinal disorder	0	1
Gastrointestinal pain	0	2
Increased appetite	0	1
Oral fungal infection	0	1
Pain in extremity	0	1

TEAE related to investigational product is defined as an AE that is 'probably related' or 'related' to the treatment.
AE, adverse event; OEC, oral enzyme combination; TEAE, treatment-emergent AE.

data demonstrating that OEC consumption triggers the formation of intermediate forms of A2M.[20] Whether this systemic increase contributes to the clinical effects of OEC in OA is unknown, as we did not detect reductions in key pro-inflammatory cytokines nor did we measure intra-articular A2M levels. Moreover, the role of A2M in OA itself is unclear, as evidence indicating that A2M levels are reduced in synovial fluid or serum is lacking.[31] However, it is thought to protect cartilage by binding to inflammatory cytokines and reducing levels of cartilage catabolic cytokines and enzymes including IL-1β, TNF-α and matrix metalloproteinases (MMPs).[31] In fact, a fabricated form of simplified A2M has been investigated as a potential TNF-α inhibitor.[32] While the numeric increase in A2M may seem modest, its statistical significance and biological role in immune regulation suggest that it could have meaningful implications for the treatment outcomes in the context of this study. Further research may be needed to explore the clinical significance of this change in A2M levels.

The significant increase in blood IL-10 levels relative to placebo following OEC treatment reported here is novel and indicates a potential systemic anti-inflammatory effect of OEC. IL-10, which has both anti-inflammatory and chondroprotective effects, is currently a target for gene therapy in OA.[33 34] IL-10 in vitro can increase proteoglycan synthesis and decrease excess production of IL-1β and TNFα.[34] As TNFα is a prototypical pro-inflammatory cytokine, TNFα and IL-10 maintain a pro-inflammatory vs anti-inflammatory cytokine balance.[35] Accordingly, in patients at risk of developing knee OA secondary to ligament trauma, circulating IL-10 concentrations are compromised.[35] Moreover, compared with patients without OA, the regulatory T cells of patients with OA secrete IL-10 at reduced amounts, and patients at more advanced stages of the disease display further reductions in IL-10 levels.[36]

uCTXII is an established biological marker for OA that reflects cartilage breakdown.[37] CTXII, a fragment originating from the telopeptide region of type II collagen, diffuses from the joint into the blood and is excreted through urine.[38 39] Higher uCTXII levels are significantly associated with risk of hand, hip and knee OA; the incidence and progression of knee OA; and clinical symptoms and radiographic findings in knee OA.[37 39 40] Its levels decrease following therapies such as intra-articular corticosteroid injections, risedronate, calcitonin or strontium ranelate.[38 39] The reductions in uCTXII observed here suggest that OEC treatment reduces cartilage turnover, although we did not detect changes in other cartilage metabolism biomarkers. Given that uCTXII release involves a different set of MMPs than those of other type II collagen peptides such as Coll2-1, our results suggest that OEC may act differentially on distinct collagen degradation pathways and/or MMPs.[41 42]

TGF-β2 and -β3 are essential growth factors for articular cartilage homeostasis, and their synthesis is dysregulated in OA.[43 44] Accordingly, the significant effects in TGF-β2 and TGF-β3 reported in OEC-treated patients here may reflect a restoration of cartilage homeostasis in OA.

Improvements in the KOOS Pain and Symptoms subdomains were found in patients treated with OEC. The Pain subdomain includes information regarding the frequency and severity of pain experienced by patients during certain activities (eg, walking, bending their knee, standing upright).[26] By contrast, we did not detect OEC-related improvements in patient-reported pain VAS scores. These null results may be due to the small sample size, which may not have provided enough power to capture the minimally clinically important difference, considered to be 17 –28 mm, in VAS.[45] The observed improvements in the KOOS Sports/Recreation subdomain, which reflects the degree of difficulty in squatting, running, jumping and turning/pivoting on the injured knee,[26] and the increased use of rescue analgesics in the placebo group suggest that patients treated with OEC increased their activity levels without the need for these drugs. This observation is crucial, given the serious side effects of these drugs and the importance of physical activity in managing chronic pain.[2 12 13]

OEC was generally well tolerated, with no serious related AEs reported in either OEC or placebo. The overall safety and tolerability profile of OEC is consistent with previous reports, with the most frequent TEAEs being gastrointestinal in nature.[15 16 19]

Limitations

This trial was exploratory in nature and aimed to explore potential biological mechanisms of OEC in patients with OA.

We have therefore chosen to use an established biomarker, linked to pathophysiology of OA, to inform sample size considerations for this trial. As such, the study was not formally powered but had a sample size estimation based on a difference of 10% in Coll2-1NO2 values. To underscore our prestudy approach, we have performed a post hoc power calculation for the observed effect of OEC on IL-10, a key finding of this study. Based on the present study sample size and the difference in IL-10 between OEC and placebo, our study has 91% power to detect the observed effect size. Nevertheless, the study could be considered too small to detect effects on several clinical parameters, such as knee pain VAS as discussed above. In addition, we did not correct for multiple testing, which, in an adequately powered trial, would have been required to assess multiple endpoints. Finally, the clinical significance of the changes in A2M, IL-10, TGF-β2 and TGF-β3 levels is unclear.

Despite randomisation, patients between the two intervention sequences had significantly different knee pain VAS at walking at the first baseline. Moreover, KOOS global scores were lower in patients assigned to OEC/placebo versus those in placebo/OEC, although this difference was not statistically significant. Whether these slight differences in knee pain VAS and/or KOOS global scores impacted the results despite the crossover design is

unknown as various knee OA phenotypes and endotypes may respond differently to treatment,[46] which we did not explicitly account for. This possibility is further emphasised by the results from the exploratory analysis, in which patients were stratified according to initial hsCRP levels, and the overall effects of OEC on IL-10 appeared to be mainly driven by patients with high baseline inflammation. However, caution should be taken when interpreting results from this subgroup analysis due to the small sample sizes.

Finally, this study was performed in patients with knee OA, and although many similarities exist in their pathogenesis, caution should be taken when extrapolating the findings from this study to hand or hip OA.[47]

Future studies

The findings of this study suggest potential novel mechanisms of action of OEC in OA, and future studies may elucidate how these translate into clinical benefits. Further research should evaluate the role of A2M and IL-10 in OA and investigate whether OEC-induced elevations in TGF-β levels confer a protective effect in OA. Additionally, the net effects of increased A2M, IL-10 and TGF-β levels on joint tissue structure should be confirmed by MRI investigation.

We performed exploratory analyses to provide insights into which OA patient subgroups would benefit the most from OEC treatment. A large international initiative to explore OA phenotypes using clinical, imaging and biochemical markers is underway, and models to predict individuals whose OA will progress are encouraging.[48 49] Our finding that baseline hsCRP may predict immunological responses (ie, IL-10 levels) to OEC may be clinically significant and should be further explored in patients whose OA is also likely to progress.

A key question is the impact of OEC on circulating pro-inflammatory cytokines as we did not observe changes in their levels following OEC treatment. Given that trypsin is endogenously produced by the pancreas, the effects of OEC reported here raise the question of whether trypsin levels are altered in knee OA.[50] Evaluating trypsin levels and/or its activity in patients with knee OA could help answer this question.

Appropriately powered studies for individual biomarkers could clarify if the absence of statistically significant differences between OEC and placebo in this study is due to sample size limitations or the lack of a biological effect. Finally, the reduction in uCTXII that we observed may indicate disease modification, but this reduction should be further investigated through long-term clinical trials.

CONCLUSIONS

This exploratory study identified biological effects of OEC that contribute to explain its potential clinical efficacy, adding to the literature the anti-inflammatory effect of OEC in OA with proposed potential mechanisms of action through IL-10 and TGF-β. The association between OEC treatment and reduced uCTXII suggests an effect on cartilage breakdown, although further confirmation is needed. The reported improvements in KOOS and the placebo-like safety profile of OEC highlight the promising clinical efficacy of OEC therapy in reducing pain and improving daily functioning in patients with OA. Larger, long-term clinical trials are required to explore if OEC could be a disease-modifying therapy for knee OA and which individuals are more likely to respond to OEC therapy.

Author affiliations
[1]musculoSKeletal Innovative Research Lab (mSKIL), Université de Liège, Liège, Belgium
[2]Institute of Musculoskeletal Medicine, University Hospital Münster, Münster, Germany
[3]Department of Geriatrics, Universitair Ziekenhuis Brussel, Brussels, Belgium
[4]Department of Rheumatology, CHU Brugmann, Brussels, Belgium
[5]Cliniques universitaires Saint-Luc, Brussels, Belgium
[6]Centre Hospitalier Régional de la Citadelle, Liège, Belgium
[7]Nestle Health Science SA, Vevey, Switzerland
[8]Ziekenhuisnetwerk Antwerpen vzw, Antwerp, Belgium
[9]Campus Middelheim, Ziekenhuis aan de Stroom, Antwerp, Belgium

Acknowledgements The authors acknowledge Ananya Das, Constantinos Bezos of Porterhouse Medical UK and Amy Chee of Porterhouse Medical US for medical writing support, which was funded by Nestlé Health Sciences, and Ismaïl Aouadi, Nestlé Health Science, for the statistical evaluation.

Contributors YH was involved in the conception and design of the study, analysis and interpretation of the results, drafting, critical revision and final approval of the article. TP was involved in the analysis and interpretation of the results, drafting, critical revision and final approval of the article. SL, VB, J-ED, DU-C and KB were involved in the provision of study materials or patients, in the collection and assembly of data, in critical revision and final approval of the article. MvE, OEJ and SR were involved in the conception and design of the study, analysis and interpretation of the results, drafting, critical revision and final approval of the article. All authors had full access to all the data in the study and had final responsibility for the decision to submit for publication. YH is guarantor for the overall content of the manuscript.

Funding The trial and investigational product were fully sponsored by Nestlé Health Science. The Sponsor was involved in the study design; participated in data collection, analysis and interpretation; funded medical writing support and agreed to submit the manuscript for publication.

Competing interests YH, consulting and speaker fees from Allegro, Artialis, Grünenthal, KiOmed Pharma, Laboratoires Expanscience, Labrha, Nestlé Health Science and Tilman. TP, consulting and speaker fees from AbbVie, Novartis, Nestlé Health Science, Peptinov and UCB. SL, no competing interests. VB, no competing interests. J-ED, no competing interests. DU-C, no competing interests. MvE, OEJ and SR are employees of Nestlé Health Sciences. KB, no competing interests.

Patient and public involvement statement All patients signed an informed consent form prior to participation in the study.

Patient consent for publication Not applicable.

Ethics approval The study was approved by the ethics committees of the participating centres (Central Ethics Committee: Comité d'Ethique Hospitalier du CHU Brugmann (077, OM 026) - CE 2020/169, Local Ethics Committees: COMMISSIE MEDISCHE ETHIEK (143, OG. 016), Reflectiegroep Biomedische Ethiek UZ Brussel; Comité d'Éthique du Centre hospitalier regional de la Citadelle (412, OM 024); COMMISSIE VOOR MEDISCHE ETHIEK ZNA (009, OG 031); Cliniques Universitaires Saint Luc UCL EC (403, OM 003). IRB: Federal Agency for Medicines and Health Products, Avenue Galilée 5/03, 1210 BRUSSELS). It was conducted in full compliance with the International Council for Harmonisation Good Clinical Practice Guidelines, the principles of the Declaration of Helsinki, and the laws and regulations of Belgium.

Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement Data are available on reasonable request.

ORCID iDs
Yves Henrotin http://orcid.org/0000-0003-1073-449X
Thomas Pap http://orcid.org/0000-0001-6514-0416
Maximilian von Eynatten http://orcid.org/0009-0006-8443-7323
Odd Erik Johansen http://orcid.org/0000-0003-2470-0530
Stefanie Rau http://orcid.org/0000-0002-9872-2399

REFERENCES

1 Giorgino R, Albano D, Fusco S, *et al*. Knee Osteoarthritis: Epidemiology, Pathogenesis, and Mesenchymal Stem Cells: What Else Is New? An Update. *Int J Mol Sci* 2023;24:6405.
2 Katz JN, Arant KR, Loeser RF. Diagnosis and Treatment of Hip and Knee Osteoarthritis: A Review. *JAMA* 2021;325:568–78.
3 Taniguchi M, Fukumoto Y, Yagi M, *et al*. Enhanced echo intensity in vastus medialis is associated with worsening of functional disabilities and symptoms in patients with knee osteoarthritis: a 3 years longitudinal study. *Rheumatol Int* 2023;43:953–60.
4 Steinmetz JD, Culbreth GT, Haile LM, *et al*. Global, regional, and national burden of osteoarthritis, 1990–2020 and projections to 2050: a systematic analysis for the Global Burden of Disease Study 2021. *Lancet Rheumatology* 2023;5:e508–22.
5 Dell'Isola A, Allan R, Smith SL, *et al*. Identification of clinical phenotypes in knee osteoarthritis: a systematic review of the literature. *BMC Musculoskelet Disord* 2016;17:425.
6 Leifer VP, Katz JN, Losina E. The burden of OA-health services and economics. *Osteoarthr Cartil* 2022;30:10–6.
7 Jang S, Lee K, Ju JH. Recent Updates of Diagnosis, Pathophysiology, and Treatment on Osteoarthritis of the Knee. *Int J Mol Sci* 2021;22:2619.
8 Bannuru RR, Osani MC, Vaysbrot EE, *et al*. OARSI guidelines for the non-surgical management of knee, hip, and polyarticular osteoarthritis. *Osteoarthr Cartil* 2019;27:1578–89.
9 Kolasinski SL, Neogi T, Hochberg MC, *et al*. 2019 American College of Rheumatology/Arthritis Foundation Guideline for the Management of Osteoarthritis of the Hand, Hip, and Knee. *Arthritis Care Res (Hoboken)* 2020;72:149–62.
10 AAOS. Management of osteoarthritis of the knee (non-arthroplasty) evidence-based clinical practice guideline (3rd edition). 2021. Available: https://www.aaos.org/oak3cpg
11 Moseng T, Vliet Vlieland TPM, Battista S, *et al*. EULAR recommendations for the non-pharmacological core management of hip and knee osteoarthritis: 2023 update. *Ann Rheum Dis* 2024;83:730–40.
12 Adebajo A. Non-steroidal anti-inflammatory drugs for the treatment of pain and immobility-associated osteoarthritis: consensus guidance for primary care. *BMC Fam Pract* 2012;13:23.
13 Henrotin YE, Michlmayr C, Rau SM, *et al*. Combination of Enzymes and Rutin to Manage Osteoarthritis Symptoms: Lessons from a Narrative Review of the Literature. *Rheumatol Ther* 2022;9:1305–27.
14 Tilwe GH, Beria S, Turakhia NH, *et al*. Efficacy and tolerability of oral enzyme therapy as compared to diclofenac in active osteoarthrosis of knee joint: an open randomized controlled clinical trial. *J Assoc Physicians India* 2001;49:617–21.
15 Bolten WW, Glade MJ, Raum S, *et al*. The safety and efficacy of an enzyme combination in managing knee osteoarthritis pain in adults: a randomized, double-blind, placebo-controlled trial. *Arthritis* 2015;2015:251521.
16 Akhtar NM, Naseer R, Farooqi AZ, *et al*. Oral enzyme combination versus diclofenac in the treatment of osteoarthritis of the knee--a double-blind prospective randomized study. *Clin Rheumatol* 2004;23:410–5.
17 Singer F, Singer C, Oberleiterner H. Phlogenzym® versus diclofenac in the treatment of activated osteoarthritis of the knee. A double-blind prospective randomized study. *Int J Immunother* 2001;17:135–41.
18 Klein G, Kullich W. Short-Term Treatment of Painful Osteoarthritis of the Knee with Oral Enzymes. *Clin Drug Investig* 2000;19:15–23.
19 Ueberall MA, Mueller-Schwefe GH, Wigand R, *et al*. Efficacy, tolerability, and safety of an oral enzyme combination vs diclofenac in osteoarthritis of the knee: results of an individual patient-level pooled reanalysis of data from six randomized controlled trials. *J Pain Res* 2016;9:941–61.
20 Lauer D, Müller R, Cott C, *et al*. Modulation of growth factor binding properties of alpha2-macroglobulin by enzyme therapy. *Cancer Chemother Pharmacol* 2001;47 Suppl:S4–9.
21 Cater JH, Wilson MR, Wyatt AR. Alpha-2-Macroglobulin, a Hypochlorite-Regulated Chaperone and Immune System Modulator. *Oxid Med Cell Longev* 2019;2019:5410657.
22 Lagrange J, Lecompte T, Knopp T, *et al*. Alpha-2-macroglobulin in hemostasis and thrombosis: An underestimated old double-edged sword. *J Thromb Haemost* 2022;20:806–15.
23 Muvhulawa N, Dludla PV, Ziqubu K, *et al*. Rutin ameliorates inflammation and improves metabolic function: A comprehensive analysis of scientific literature. *Pharmacol Res* 2022;178:106163.
24 Horcajada M-N, Sanchez C, Membrez Scalfo F, *et al*. Oleuropein or rutin consumption decreases the spontaneous development of osteoarthritis in the Hartley guinea pig. *Osteoarthr Cartil* 2015;23:94–102.
25 Na JY, Song K, Kim S, *et al*. Rutin protects rat articular chondrocytes against oxidative stress induced by hydrogen peroxide through SIRT1 activation. *Biochem Biophys Res Commun* 2016;473:1301–8.
26 Roos EM, Roos HP, Lohmander LS, *et al*. Knee Injury and Osteoarthritis Outcome Score (KOOS)--development of a self-administered outcome measure. *J Orthop Sports Phys Ther* 1998;28:88–96.
27 Henrotin Y, Bannuru R, Malaise M, *et al*. Hyaluronan derivative HYMOVIS® increases cartilage volume and type ii collagen turnover in osteoarhritic knee: data from MOKHA study. *BMC Musculoskelet Disord* 2019;20:293.
28 Schoenthaler M, Miernik A, Offner K, *et al*. The cumulative analgesic consumption score (CACS): evaluation of a new score to describe postsurgical analgesic consumption as a surrogate parameter for postoperative pain and invasiveness of surgical procedures. *Int Braz J Urol* 2014;40:330–6.
29 Hick A-C, Malaise M, Loeuille D, *et al*. Cartilage Biomarkers Coll2-1 and Coll2-1NO2 Are Associated with Knee OA MRI Features and Are Helpful in Identifying Patients at Risk of Disease Worsening. *Cartilage* 2021;13:1637S–1647S.
30 Mobasheri A, Lambert C, Henrotin Y. Coll2-1 and Coll2-1NO2 as exemplars of collagen extracellular matrix turnover - biomarkers to facilitate the treatment of osteoarthritis? *Expert Rev Mol Diagn* 2019;19:803–12.
31 Wang S, Wei X, Zhou J, *et al*. Identification of α2-macroglobulin as a master inhibitor of cartilage-degrading factors that attenuates the progression of posttraumatic osteoarthritis. *Arthritis Rheumatol* 2014;66:1843–53.
32 Zheng W, Zhou T, Zhang Y, *et al*. Simplified α_2-macroglobulin as a TNF-α inhibitor for inflammation alleviation in osteoarthritis and myocardial infarction therapy. *Biomaterials* 2023;301.
33 Evans CH, Ghivizzani SC, Robbins PD. Osteoarthritis gene therapy in 2022. *Curr Opin Rheumatol* 2023;35:37–43.
34 Wojdasiewicz P, Poniatowski ŁA, Szukiewicz D. The role of inflammatory and anti-inflammatory cytokines in the pathogenesis of osteoarthritis. *Mediators Inflamm* 2014;2014:561459.
35 Barker T, Rogers VE, Henriksen VT, *et al*. Circulating IL-10 is compromised in patients predisposed to developing and in patients with severe knee osteoarthritis. *Sci Rep* 2021;11:1812.
36 Li S, Wan J, Anderson W, *et al*. Downregulation of IL-10 secretion by Treg cells in osteoarthritis is associated with a reduction in Tim-3 expression. *Biomed Pharmacother* 2016;79:159–65.
37 Valdes AM, Meulenbelt I, Chassaing E, *et al*. Large scale meta-analysis of urinary C-terminal telopeptide, serum cartilage oligomeric protein and matrix metalloprotease degraded type II collagen and their role in prevalence, incidence and progression of osteoarthritis. *Osteoarthr Cartil* 2014;22:683–9.
38 Klocke R, Levasseur K, Kitas GD, *et al*. Cartilage turnover and intra-articular corticosteroid injections in knee osteoarthritis. *Rheumatol Int* 2018;38:455–9.
39 Rousseau J-C, Chapurlat R, Garnero P. Soluble biological markers in osteoarthritis. *Ther Adv Musculoskelet Dis* 2021;13:1759720X211040300.

40 Liem Y, Judge A, Kirwan J, *et al*. Multivariable logistic and linear regression models for identification of clinically useful biomarkers for osteoarthritis. *Sci Rep* 2020;10:11328.
41 Henrotin Y, Addison S, Kraus V, *et al*. Type II collagen markers in osteoarthritis: what do they indicate? *Curr Opin Rheumatol* 2007;19:444–50.
42 Huebner JL, Williams JM, Deberg M, *et al*. Collagen fibril disruption occurs early in primary guinea pig knee osteoarthritis. *Osteoarthr Cartil* 2010;18:397–405.
43 Duan M, Wang Q, Liu Y, *et al*. The role of TGF-β2 in cartilage development and diseases. *Bone Joint Res* 2021;10:474–87.
44 Du X, Cai L, Xie J, *et al*. The role of TGF-beta3 in cartilage development and osteoarthritis. *Bone Res* 2023;11:2.
45 Bird SB, Dickson EW. Clinically significant changes in pain along the visual analog scale. *Ann Emerg Med* 2001;38:639–43.
46 Roemer FW, Jarraya M, Collins JE, *et al*. Structural phenotypes of knee osteoarthritis: potential clinical and research relevance. *Skeletal Radiol* 2023;52:2021–30.
47 He Y, Li Z, Alexander PG, *et al*. Pathogenesis of Osteoarthritis: Risk Factors, Regulatory Pathways in Chondrocytes, and Experimental Models. *Biology (Basel)* 2020;9:194.
48 van Helvoort EM, van Spil WE, Jansen MP, *et al*. Cohort profile: The Applied Public-Private Research enabling OsteoArthritis Clinical Headway (IMI-APPROACH) study: a 2-year, European, cohort study to describe, validate and predict phenotypes of osteoarthritis using clinical, imaging and biochemical markers. *BMJ Open* 2020;10:e035101.
49 van Helvoort EM, Jansen MP, Marijnissen ACA, *et al*. Predicted and actual 2-year structural and pain progression in the IMI-APPROACH knee osteoarthritis cohort. *Rheumatology (Oxford)* 2022;62:147–57.
50 Logsdon C. Phosphatidylinositol 3-kinase and trypsin activation in pancreatitis. *J Clin Invest* 2001;108:1267–8.