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Oleuropein or rutin consumption decreases the spontaneous development of osteoarthritis in the Hartley guinea pig

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SUMMARY

Objective: To assess the potential protective effects of three polyphenols oleuropein, rutin and curcumin, on joint ageing and osteoarthritis (OA) development.
Design: Sixty 4-week-old Dunkin–Hartley guinea pigs were randomized into four groups and received daily during 31 weeks either standard guinea pig diet (control group) or a standard guinea pig diet enriched with oleuropein (0.025%), rutin (0.5%) or rutin/curcumin (0.5%/0.25%) association. Biomarkers of OA (Coll2-1, Coll2-1NO2, Fib3-1, Fib3-2, ARGS), as well as inflammation prostaglandin E_2 (PGE_2) were quantified in the serum. Histological assessments of knee cartilage and synovial membrane were performed at week 4 (five young reference guinea pigs) and week 35.
Results: At week 35, guinea pigs in the control group spontaneously developed significant cartilage lesions with mild synovial inflammation. The histological scores of cartilage lesions and synovitis were well correlated with the increased level of serum biomarkers. Histologically, all treatments significantly reduced the cartilage degradation score ($P < 0.01$), but only oleuropein significantly decreased the synovial histological score ($P < 0.05$) and serum PGE_2 levels ($P < 0.01$) compared to the control group. Coll2-1 was decreased by rutin and the combination of rutin/curcumin, Fib3-1 and Fib3-2 were only decreased by the rutin/curcumin mixture, while Coll2-1NO2 was significantly decreased by all treatments ($P < 0.05$).
Conclusion: Oleuropein and rutin ± curcumin significantly slowed down the progression of spontaneous OA lesions in guinea pigs. While no additive effect was seen in the curcumin + rutin group, the differential effects of oleuropein and rutin on inflammatory and cartilage catabolic markers suggest an interesting combination for future studies in OA protection.

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Introduction

Osteoarthritis (OA) is a high prevalence disease with an important socio-economic impact. OA is characterized by fibrillations, fissures, and even in late stage of the disease, disappearance of cartilage. Furthermore, cartilage degradation is associated with structural and metabolic changes in other joint tissues such as subchondral bone sclerosis and synovial membrane inflammation[1]. Numerous models have been proposed to investigate the natural course of the disease and to study the effects of treatments. Among these models, Dunkin–Hartley guinea pigs developing spontaneous OA is very attractive because

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it mimics well the pathophysiological processes observed in primary human OA[2].

The appearance of joint pathology in both guinea pigs and humans is age-related and subject to a variety of well-recognized OA risk factors including body weight, ligament laxity and high bone turnover. Spontaneous lesions in the knee are bilateral and are more pronounced in the medial compartment in the area not covered by the meniscus. Bone cysts (2–3 months old), subchondral bone thickening and osteophytes (3–12 months old) are preceded by type II collagen fibril disruption (2 months old)[3], histological proteoglycan loss (4–6 months old) and cartilage fibrillations (8–12 months old)[2,4–7]. The involvement of interleukin (IL)-1β and matrix metalloproteinases (MMP-3 and-13) in this degenerative process has been shown[8].

Some epidemiological studies have reported an association between long-term consumption of diets rich in polyphenols and protection against chronic diseases[9] but few studies have investigated the effects of such compounds on cartilage and no long-term studies on OA have been carried out with phytonutrients[10,11]. Nevertheless, antioxidant and anti-inflammatory properties of polyphenols make them interesting candidates to study their potential protective effect on cartilage. Recently, data from clinical studies suggest that curcumin improved joint function, reduced pain and type II collagen degradation in OA patients[12,13].

The leaves and fruit of the olive plant (*Olea europaea* L.) are rich in polyphenols exhibiting a range of beneficial effects including antioxidant, anti-inflammatory, antiatherogenic and anticarcinogenic properties[14], the most bioactive ones being oleuropein and hydroxytyrosol. It has recently been demonstrated that oleuropein protects against collagen II-induced arthritis in mice[15]. Rutin (quercetin-3-O-rutinoside) is a flavonoid ubiquitously found in plants. Quercetin, the circulating aglycone form of rutin, is considered to be a strong antioxidant due to its ability to scavenge free radicals[16,17].

The aim of the current study was to investigate if these polyphenols would exert a protective effect on cartilage and influence the natural course of spontaneous OA in Dunkin–Hartley guinea pigs. Primary outcome was the improvement of global OA histological score, and secondary outcomes were the variation of serum levels of biomarkers prostaglandin E_2, fibulin-3 fragments (Fib3-1 and Fib3-2), collagen (Coll2-1 and Coll2-1NO2) and aggrecan (ARGS) neoepitopes.

Methods

All experimental procedures and protocols used in this investigation were reviewed and approved by the Institutional Animal Care and Use Ethics Committee of the University of Liège (Belgium), reference 1207. The "Guide for the Care and Use of Laboratory Animals," prepared by the Institute of Laboratory Animal Resources, National Research Council, and published by the National Academy Press, was followed carefully as well as European and local legislation. Sixty-five 3-week-old male Dunkin–Hartley guinea pigs were obtained from Charles River Laboratories (Paris, France). Identification was made by microchip. Animals were housed five per solid bottom cages and fed with a standard guinea pig chow diet (Table I) and water *ad libitum*. PVC pipes were added to the cages to improve housing conditions and minimize stress. All animals allowed 10 days for acclimatization to housing conditions prior to phytonutrient administration. As young reference group, five guinea pigs were sacrificed at 4-week-old. The 60 remaining male 4-week-old Hartley guinea pigs were randomized into four groups: one receiving a diet containing a standard chow diet ($n = 15$, the control group) and the other groups receiving either a standard chow diet enriched with 0.025% of oleuropein ($n = 15$), or with 0.5% of rutin ($n = 15$) or with 0.5% of rutin and 0.25% of curcumin ($n = 15$) until week 35 (Table I). The daily dose consumed during 31 weeks corresponds approximately to an intake of 12.5 mg/kg body weight for oleuropein, 250 mg/kg for rutin (thus 125 mg total equivalent aglycon) and 125 mg/kg for curcumin. The number of animal per group was choose according to the Osteoarthritis Research Society International (OARSI) recommendation[18] to provide 80% power to detect a significant change in response to a hypothetical treatment capable of achieving a 30% treatment difference compared to untreated animals, with statistical significance of $P = 0.05$.

Table I
Composition of the experimental diets

	Control in (%)	Oleuropein (%)	Rutin (%)	Rutin + curcumin (%)
Corn starch*	46.4	46.38	45.9	45.65
Soy protein†	18.7	18.7	18.7	18.7
DL-methionine‡	1.0	1.0	1.0	1.0
Sucrose§	5.0	5.0	5.0	5.0
Cellulose‖	10.0	10.0	10.0	10.0
Guar gum‡	2.5	2.5	2.5	2.5
Mineral mix¶	7.5	7.5	7.5	7.5
Vitamin mix¶	1.0	1.0	1.0	1.0
Choline bitartrate‡	0.5	0.5	0.5	0.5
Ascorbic acid‡	0.4	0.4	0.4	0.4
Kliba diet 3420#	3.0	3.0	3.0	3.0
Palm oil‡	0.8	0.8	0.8	0.8
Soybean oil**	3.04	3.04	3.04	3.04
Linseed oil‡	0.16	0.16	0.16	0.16
Oleuropein††	0	0.025	0	0
Rutin‡	0	0	0.5	0.5
Curcumin‡	0	0	0	0.25

†† Extrasynthèse, Genay, France.
* Schweizerhall Chemie, Basel, Switzerland.
† Solae, Grand-Saconnex, Switzerland.
‡ Sigma Aldrich, Buchs, Switzerland.
§ Howeg, Aaberg, Switzerland.
‖ Christ Water Technology Group, Basel, Switzerland.
¶ Vital AG, Oberentfelden, Switzerland according to mineral (no250001) and vitamin (no 350001) mix composition from Dyets, Inc., Bethlehem, PA.
\# Provimi Kliba AG, Kaiseraugst, Switzerland.
** Florin, Muttenz, Switzerland.

Animals were weighed every week. Food intake was controlled at weeks 9, -15, -21, -26 and -34.

Blood sampling

At week 4, -10, -16, -22 and -28, 500 μl of blood was collected at the superficial veins of the ears, in the morning, under ketamine (32 mg/kg)/xylazine (3 mg/kg) subcutaneous anaesthesia.

At week 4 for the young reference group and at week 35 for the other groups, 2 ml of blood was collected by intracardiac puncture, under general anaesthesia (sodium pentobarbital 200 mg/kg, intraperitonealy), just before dead.

Blood was then centrifuged at 2000× *g* for 5 min, and serum stored at −80°C until analysis.

Histology

At euthanasia (week 4 or 35), the right knee joint from each animal was fixed for 24 h in 4% buffered paraformaldehyde, followed by decalcification in HCl acid (DC2, Labonord) for 4 h at 4°C before paraffin embedding. The right kidney and a piece of liver were also fixed in 4% buffered paraformaldehyde and paraffin embedded.

Paraffin embedded right knees were cut with a microtome into 6 μm sections, in the central area not covered by meniscus following the Cushin plane, as recommended by OARSI[18]. Three

sections at 200 μm intervals were stained with haematoxylin, fast green and safranin-O, or toluidine blue. To establish the OA score, each compartment of the section (tibial median, tibial lateral, femoral median and femoral lateral) was scored by two blinded trained experts following OARSI recommendations for guinea pig (cartilage surface integrity 0–8, proteoglycan content 0–6, cellularity 0–3, tidemark integrity 0–1 and osteophyte 0–3, with a maximum of 21 per compartment). The mean of three sections score were calculated for each knee. To assess the global OA score (primary outcome), scores of each compartments were added, giving a maximal score of 84. Lateral and medial synovial membranes were also scored (synovial lining cells hyperplasia 0–2, villous hyperplasia 0–3, degree of cellular infiltration by perivascular lymphocytes and mononuclear cells 0–5) and the mean of lateral and median membrane was calculated to assess the global synovial score (maximum score of 10)[18].

PGE_2, ARGS, Coll2-1, Coll2-1NO2, Fib3-1 and Fib3-2 immunoassays

Prostaglandin E_2 (PGE_2) was measured using a competitive ELISA kit (Arbor Assays, USA), in serum collected at sacrifice (week 4 in young animal controls or week 35 in the other animals).

Coll2-1, Coll2-1NO2, Fib3-1 and Fib3-2 were quantified in triplicate by competitive ELISA, and ARGS in triplicate in deglycosylated samples using a sandwich immunoassay with ECL technology on a Meso Scale Discovery (MSD) platform, derived from immunoassay previously described[19], in serum collected at veins of the ears at week 4,-10,-16,-22,-28, and intracardiacaly at week 35 (Artialis SA, Liège, Belgium).

Calculation and statistical analysis

Results are expressed as mean with 95% CI. Following a normality test (Kolmogorov–Smirnov test for biomarker kinetics and D'Agostino and Pearson omnibus normality test), one-way analysis of variance (one-way ANOVA) with Dunnett's post-test were performed for histology and biomarkers between week 4 and 35 in the control group and between treated groups and the control group at week 35 (GraphPad Prism 5.0), Pearson correlations were performed (GraphPad Prism 5.0) between each parameters. For longitudinal analysis of the biomarkers, a mixed model with an undefined covariance structure was fitted to the data to test for differences between the treatment groups. The covariates included in the model were the time and the interaction with the treatment indicator. However, based on a graphical investigation of the data, a quadratic time effect was considered for variable Fib3-2, Coll2-1, and Coll2-1NO2. This statistical method permits the comparison of response curves between treatments while accounting for repeated data within each guinea pig. Calculations were always carried out on the maximum number of data available. Results were considered to be significant at the 5% critical level ($P < 0.05$). Data analysis was carried out using SAS (version 9.3 for Windows) statistical package.

Results

Housing and weight evolution

At baseline (week 4), guinea pigs weighed 262 g (95% CI: 258, 266). The four groups gained weight in the same way during the study. No difference of weight between groups was observed during the study and food intake was similar in all groups. At the end of the study (week 35), weight of the animals was 934 g (95% CI: 910, 964). One guinea pig was excluded from the analysis in the control group because its weight was only 620 g at week 35, clearly out of range comparing to the other animals. Six guinea pigs died during the study, two in control group, two in oleuropein group and two in rutin group. Data collected until their deaths were used for the analysis. With the exception of one spontaneous death in rutin group at week 23, complications that led to other death/euthanasia were:

- peritoneal lesion after injection (one from oleuropein group at week 10).
- incapacity for two guinea pigs to wake up (two from control group at week 16).
- acute myocardial infarction after handling (one from rutin group at week 28).
- severe injuries after fight with a fellow (one from oleuropein group at week 31).

Histology

As expected, guinea pigs spontaneously developed severe knee OA (Fig. 1). In all animals, the global OA histological score increased with age [$P < 0.001$ between week 4 and 35, Fig. 2 (A)]. At week 35, a significant decrease of the global OA histological score was observed in treated group compared to controls: 46 (95% CI: 41.4, 50.6) in control vs 33.2 (95% CI: 29.9, 36.5) in oleuropein group ($P < 0.001$), vs 36.9 (95% CI; 32.9, 40.8) in rutin group ($P < 0.01$) and vs 36.7 (95% CI: 32.2, 41.1) in rutin + curcumin ($P < 0.01$) group [Figs. 1 and 2(A)]. No significant difference between the treated groups was observed. When femoral, tibial, medial or lateral compartments were analysed individually, oleuropein and rutin decreased histological lesions severity in each compartment. In contrast, rutin + curcumin tended, but didn't significantly decrease the OA histological score in the femoral compartment but was efficient in the other compartments. When histological items were analysed individually, all treatments improved significantly cartilage surface integrity [$P < 0.01$, Fig. 2(B)] and increased proteoglycan content [$P < 0.01$, Fig. 2(C)]. Furthermore, cellularity score was significantly lower in oleuropein and rutin + curcumin groups [$P < 0.05$, Fig. 2(D)], and osteophyte score significantly lower in oleuropein group compared to controls [$P < 0.05$, Fig. 2(F)]. No treatment was able to modify the tidemark integrity score [Fig. 2(E)].

A significant increase of the synovial score between week 4 and week 35 was observed in the control group [$P < 0.01$, Fig. 3(A)]. The global synovial OA histological score are well correlated with the global OA histological score ($r = 0.81$, $P < 0.001$).

At week 35, only oleuropein significantly decreased the global synovial OA histological score 4 (95% CI; 3.2, 4.8) in control group vs 2.9 (95% CI: 2.1, 3.6) in oleuropein group [$P < 0.05$, Fig. 3(A)]. More precisely, oleuropein decreased lining and infiltrated cells ($P < 0.05$), but was ineffective on villous hyperplasia (data not shown).

Serum PGE_2

From week 4 to 35, serum PGE_2 levels significantly increased in the control group (2.3-fold, $P < 0.001$) and were positively correlated with the global OA histological score ($r = 0.54$, $P < 0.05$) and the global synovial histology score ($r = 0.55$, $P < 0.05$).

At week 35, serum PGE_2 levels were decreased by 46% in the oleuropein group compared to the control group [$P < 0.01$, Fig. 3(B)]. In contrast, rutin and rutin + curcumin didn't modify PGE_2 levels.

Serum Coll2-1 and Coll2-1NO2

An overall inverted-U shape (or concave) trajectory was found for Coll2-1 and Coll2-1NO2. Serum Coll2-1 levels increased

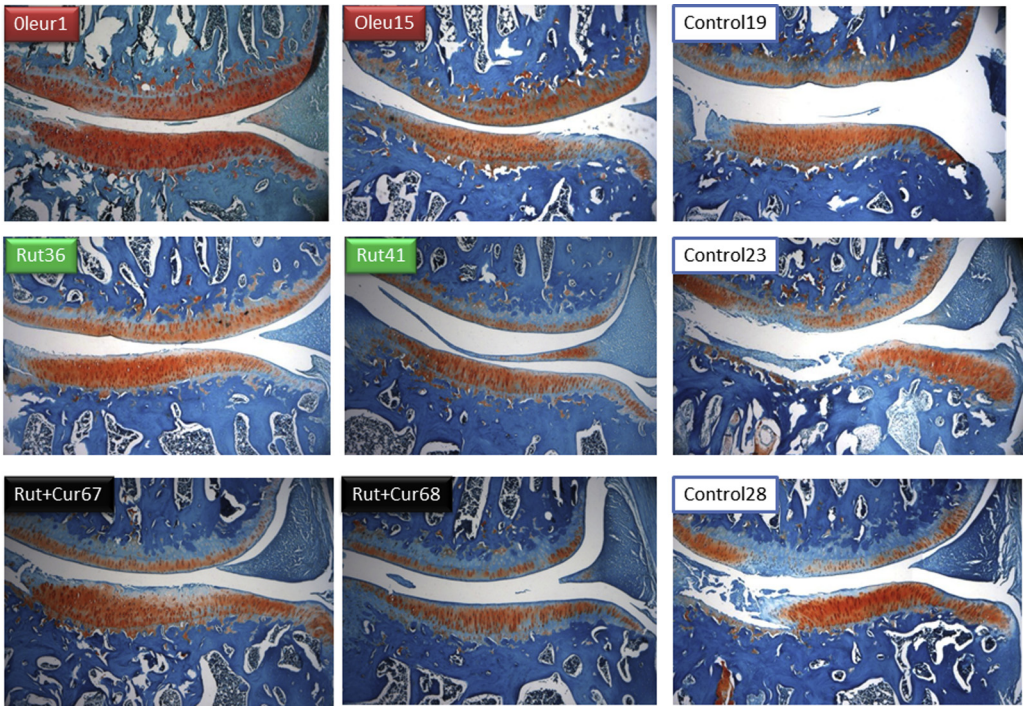

Fig. 1. Representative pictures of medial compartment of right guinea pig knees of each group, at week 35. Control = control group, Oleur = Oleuropein group, Rut = Rutin group, Rut + Cur = Rutin + curcumin group. Safranin-O/fast green/hematoxylin staining, 5× magnification.

significantly until week 16, reached a maximum at week 22 (3-fold increase compared to week 4 in the control group, $P < 0.001$) and decreased between week 28 and 35 [Fig. 4(A)]. At week 35, serum Coll2-1 level was still 1.8-fold higher than at week 4 ($P < 0.001$). Coll2-1 NO2 reached a maximum at week 28 (2-fold increase compared to week 4 in the control group, $P < 0.001$) and tended to decrease to week 35 [still 1.8-fold higher than at week 4, $P < 0.001$, Fig. 4(B)]. At week 35, no significant correlation was found between Coll2-1 serum levels and the global histological OA or synovial scores. However, a significant and positive correlation was found between the change of Coll2-1 between week 4 and 10 and the global histological OA score at week 35 in all animals ($r = 0.32$, $P < 0.05$). A strong correlation was found at week 35 between Coll2-1NO2 levels and global OA score in the control group ($r = 0.77$, $P < 0.001$), and with the synovial score ($r = 0.56$, $P < 0.05$) as well as between the evolution of Coll2-1NO2 between week 4 and 10 and the final histological OA score at week 35 in all animals ($r = 0.43$, $P < 0.01$). The Coll2-1NO2 levels quantified at week 10 ($r = 0.46$, $P < 0.001$) or at week 16 ($r = 0.36$, $P < 0.01$) were also positively correlated to the global histological OA score at week 35 in all animals.

The kinetic curves of serum Coll2-1 concentration were comparable in the oleuropein and the control group ($P = 0.75$). However, serum Coll2-1 kinetic curves of guinea pigs treated with rutin ($P = 0.0001$) or rutin + curcumin ($P = 0.0015$) were significantly lower than those of the control group [Fig. 4(A)]. The decrease was significant from week 16 ($P < 0.05$).

The Coll2-1NO2 kinetic curve in the controls was significantly higher than those of the other groups [$P < 0.0001$, Fig. 4(B)]. This effect was significant from week 10 ($P < 0.01$).

Serum Fib3-1 and Fib3-2

Fib3-1 and Fib3-2 serum levels increased over time in the control group [$P < 0.0001$, Fig. 4(C) and (D)]. Fib3-1 increased significantly steadily with time (2.5-fold increase in control group at week 35 compared to week 4), while Fib3-2 reached a maximum at week 22 (2.1-fold increase in control group compared to week 4) and then remained stable until week 35. A strong correlation was found at week 35 between Fib3-1 ($r = 0.68$, $P < 0.01$) or Fib3-2 ($r = 0.85$, $P < 0.001$) levels and the global histological OA score, and between Fib3-2 and the global synovial histological score ($r = 0.60$, $P < 0.05$).

However, while Fib3-1 kinetic curve of the oleuropein group was not statistically different of that of the control group ($P = 0.52$), the kinetic curve of rutin + curcumin group was significantly lower ($P = 0.003$), and tended to be lower in the rutin group ($P = 0.097$) than that of the control [Fig. 4(C)].

The Fib3-2 kinetic curves varied with treatments [$P = 0.0003$, Fig. 4(D)]. In fact, as compared to the control group, Fib3-2 increased more slowly in oleuropein group ($P = 0.0064$) and in rutin + curcumin group ($P = 0.0014$). No significant difference was found between rutin and control group kinetic curves ($P = 0.90$).

Serum ARGS

The serum levels of ARGS decreased significantly with time [$P < 0.001$, Fig. 5(A)] in all groups. No significant difference between kinetic curves was observed. However, at week 35, ARGS levels were significantly lower in the rutin and the rutin + curcumin groups than in the control group [$P < 0.05$, Fig. 5(B)].

Discussion

In this study, three phytonutrients, oleuropein and rutin alone or a combination of rutin and curcumin, were evaluated at physiological and nutritional doses for their effects on spontaneous development of OA in the guinea pig model.

Same doses have been shown to be efficient in rodents for different health benefits, e.g., bone health[20,21] or atherosclerosis[22,23].

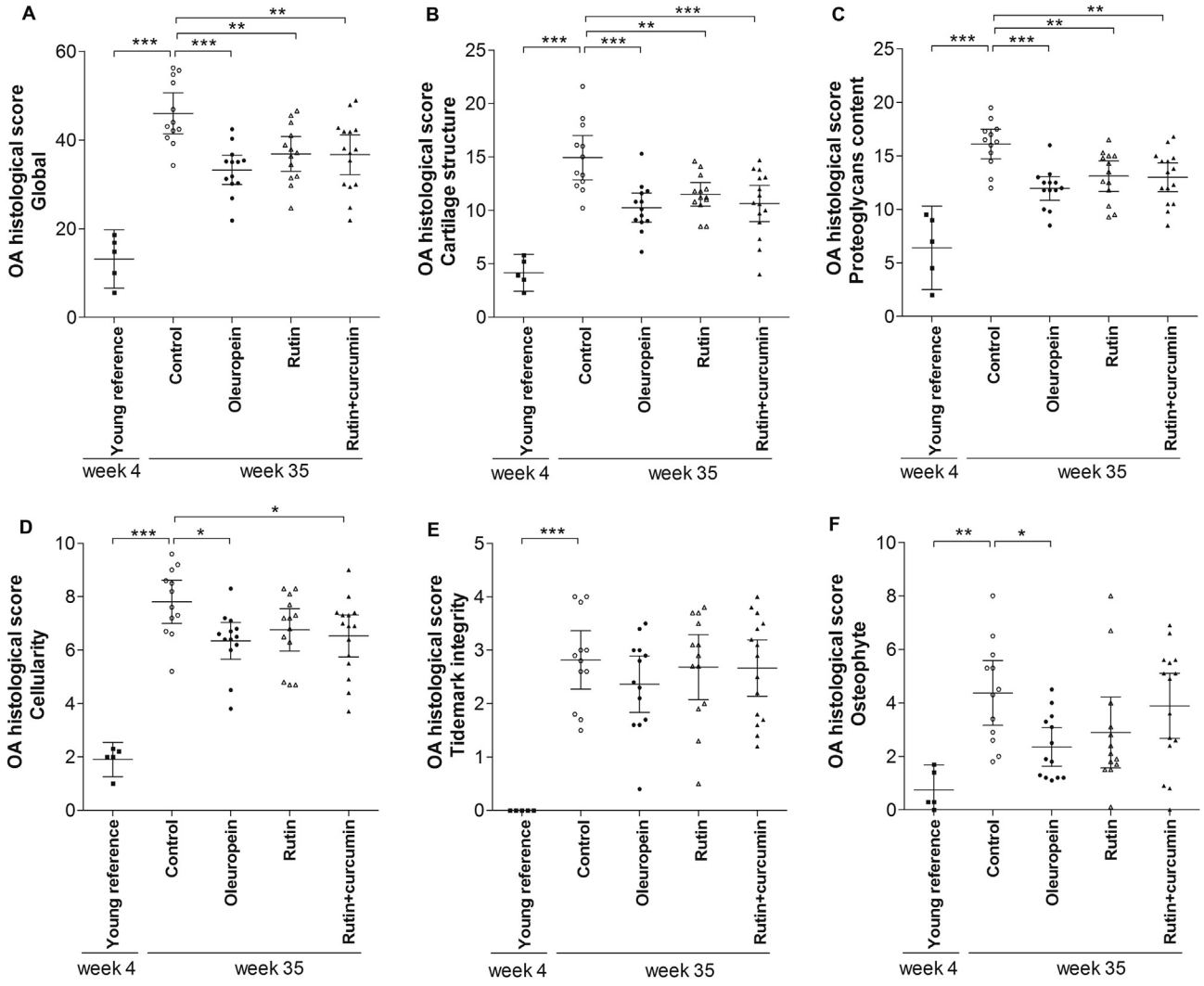

Fig. 2. (A) Global OA score. (B) Cartilage surface integrity score. (C) Proteoglycans content score. (D) Cellularity score. (E) Tidemark integrity score. (F) Osteophyte score. Mean ± 95% CI, * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$, one-way ANOVA with Dunnet's post-test.

At these dose ranges, metabolite circulating levels in rodent (as total aglycon equivalent) has been reported 9.46 ± 1 μM for rutin[20], 24 ± 2.3 nM for oleuropein[24,25] and 1.6 ± 0.1 μM for curcumin glucuronide (0.28 ± 0.07 μM for curcumin sulfate)[26].

None of the phytonutrient supplements significantly modified food intake or guinea pig growth. This important finding indicates that treatment effects were not linked to weight changes[27]. Tested compounds did not induce acute toxicity on major organs like kidney or liver (data not shown).

Supplementation with these phytonutrient treatments significantly slowing down OA development, showing a significant decrease of the histological OA scores compared to controls. Looking individually at each parameter of this global score, oleuropein and rutin reduced cartilage surface integrity and proteoglycans content scores, but only oleuropein was efficient on decreasing osteophyte formation. In this model, other treatments such as tetracyclines[28], pioglitazone[29], hyaluronan intra-articular injections[30] or phosphocitrate administration[31] have previously shown an improvement of this global Mankin-derived score.

We evaluated polyphenol effects on inflammation by measuring PGE_2 in serum and by assessing synovial histological score. Only oleuropein consumption significantly reduced these parameters, indicating that this compound acts on both cartilage lesion and synovium inflammation. Nevertheless, this remains a secondary outcome only enlightened us on mechanisms, because this spontaneous animal model shows only a mild inflammation, like in human OA.

We also aimed to study some OA biomarkers in this guinea pig model, and their relevance to follow-up treatments slowing down OA development. More precisely, we measured serum Coll2-1, Coll2-1NO2, Fib3-1 and Fib3-2, and a neoepitope of aggrecanase degradation of aggrecan, ARGS.

These biomarkers are all related to OA disease. Coll2-1, a peptide located in the triple helical part of type II collagen molecule, is a marker of collagen network degradation found at elevated levels in the serum of knee and hip OA patients[32]. In this study, Coll2-1 increased early in OA development. This finding corroborates a previous study demonstrating that Coll2-1 increase was concomitant with the early type II collagen fibril disruption[3]. Further, the change of serum Coll2-1 levels between week 4 and 10 was correlated with the final global histological OA score at week 35. This means that the early increase of Coll2-1 could be predictive of the OA natural course. Coll2-1NO2 is the nitrated form of Coll2-1 and correlated with c-reactive protein (CRP) in human OA and RA patients[33]. Fib3-1 and Fib3-2 are two specific peptides of fibulin-3.

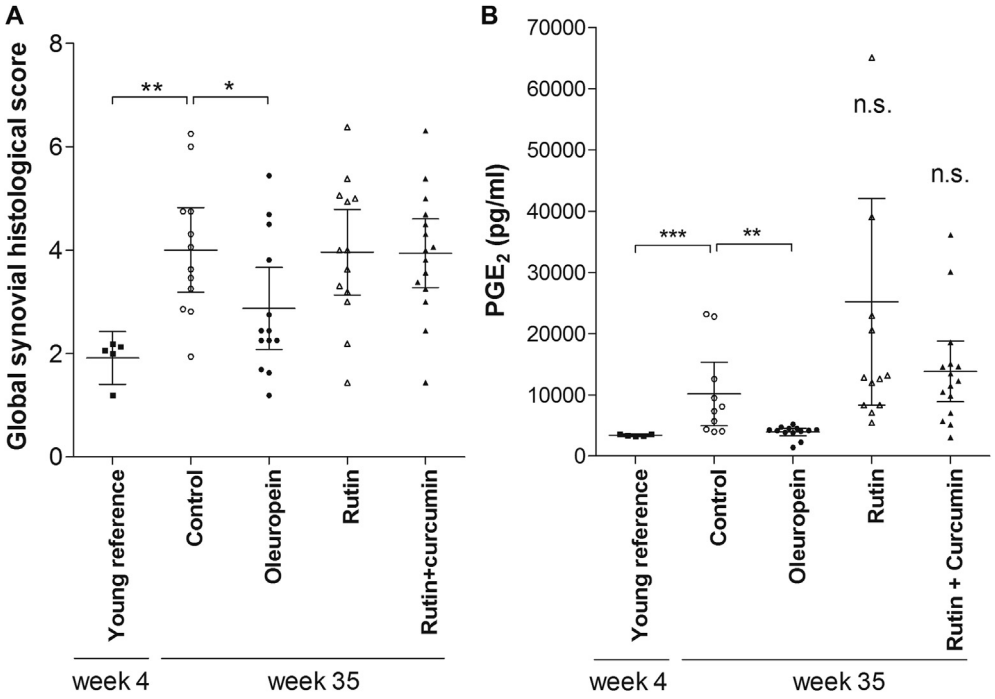

Fig. 3. (A) Global synovial histological score in each group. (B): PGE_2 levels found in guinea pig sera at week 4 or week 35. Mean ± 95% CI, * = $P < 0.05$, ** = $P < 0.01$ and *** = $P < 0.001$, one-way ANOVA with Dunnet's post-test.

These peptides were increased in the serum of OA patients compared to healthy subjects[34]. The antibodies used in the Fib3-1 and Fib3-2 immunoassays don't recognize the native fibulin-3 indicating that Fib3-1 and Fib3-2 levels reflect fibulin-3 degradation. This is important because fibulin-3 knock-out mice develop OA indicating that fibulin-3 is important to maintain cartilage homoeostasis. In this study, we showed for the first time that Coll2-1NO2, Fib3-1 and Fib3-2 were correlated with histological global OA score while Coll2-1NO2 and Fib3-2 also correlated with synovial histological score. These data suggest that these markers are relevant burden of disease biomarkers in guinea pig OA model. They may also be useful to evaluate efficacy of intervention (e.g., nutrients) since early decrease of these biomarkers was correlated with the decrease of the histological OA score induced by phytonutrients. For the first time, we have also investigated the kinetic of ARGS, a neoepitope generated by aggrecanases, in the serum of guinea pig. Serum ARGS drastically decreased between weeks 4 and 22, and tended to increase between week 28 and 35. The early drop of serum ARGS, probably reflects the high turnover in the growth plate of these guinea pigs which are still in growing phase until 18-week-old. This biomarker could be helpful after the growing phase to follow OA course in this guinea pig model. However, additional longer experiments are needed to confirm and to validate this hypothesis.

Rutin alone or with curcumin early decreased Coll2-1, Coll2-1NO2, and later ARGS, showing an anti-catabolic profile of these phytonutrients. In contrast, oleuropein decreased Coll2-1NO2 levels in parallel to the PGE_2 levels and synovial score suggesting that this compound exerts *in vivo* anti-inflammatory and antioxidant effects. This observation also indicates that oleuropein and rutin modulate different OA physiopathologic processes. This also gives a rationale to the combined administration of oleuropein and rutin to treat OA.

The ability of rutin to decrease the biomarkers of cartilage degradation as well as the cartilage lesion histological score may be related to the antioxidant properties of its metabolite quercetin. Indeed, it was recently shown that quercetin was able to decrease aggrecan loss from explants and IL-1-stimulated a disintegrin and metalloproteinase with thrombospondin motif (ADAMTS-4) in chondrocytes[35].

Surprisingly, when mixing with rutin, curcumin did not shown any additive or synergistic effect on histological or synovial scores This could be explained by the action already provide by rutin treatment in this study.

Oleuropein effects may be linked to oleuropein itself or to its major metabolite, hydroxytyrosol, which is one of the most potent oxygen radical scavenger[36] and a potent activator of manganese superoxide dismutase gene expression[37]. Hydroxytyrosol also has anti-inflammatory properties, decreasing 5-lipooxygenase activity and leukotriene B4 production. Oleuropein elicited protective effects on bone mass in a rat model in which bone loss was associated with ovariectomy and acute inflammation[21]. It was hypothesised that oleuropein may exert its bone-sparing effect by modulating inflammation rather than acting directly on bone metabolism. Indeed, neither oleuropein nor whole olive oil was able to affect bone mineral density in ovariectomised rats when inflammation was not induced[38].

The major limitation of our study is the absence of a non-OA age matching control group. This is important since we know that Hartley guinea pig bone growth is prolonged until week 18. During the first weeks, some biomarkers serum levels are probably confounded by growth plate remodelling associated with a high release of neoepitopes. However, no staining of Coll2-1 was found in the cartilage growth plate of these animals[3]. Furthermore, fibulin-3 fragment biomarkers lack of tissue specificity. Indeed, fibulin-3 is ubiquitous and found in a lot of tissues, therefore we can suspect that these biomarkers levels reflected more a systemic effect of the drugs, on different connective tissues. Another particularity of this model is that early OA lesions appear concomitantly with growth plate remodelling. For this reason, we have investigated biomarkers levels at an early time point, before the first type II collagen lesions are visible[3]. Indeed, in this study we observed that Coll2-1, Coll2-1NO2, Fib3-1 and Fib3-2 increased early

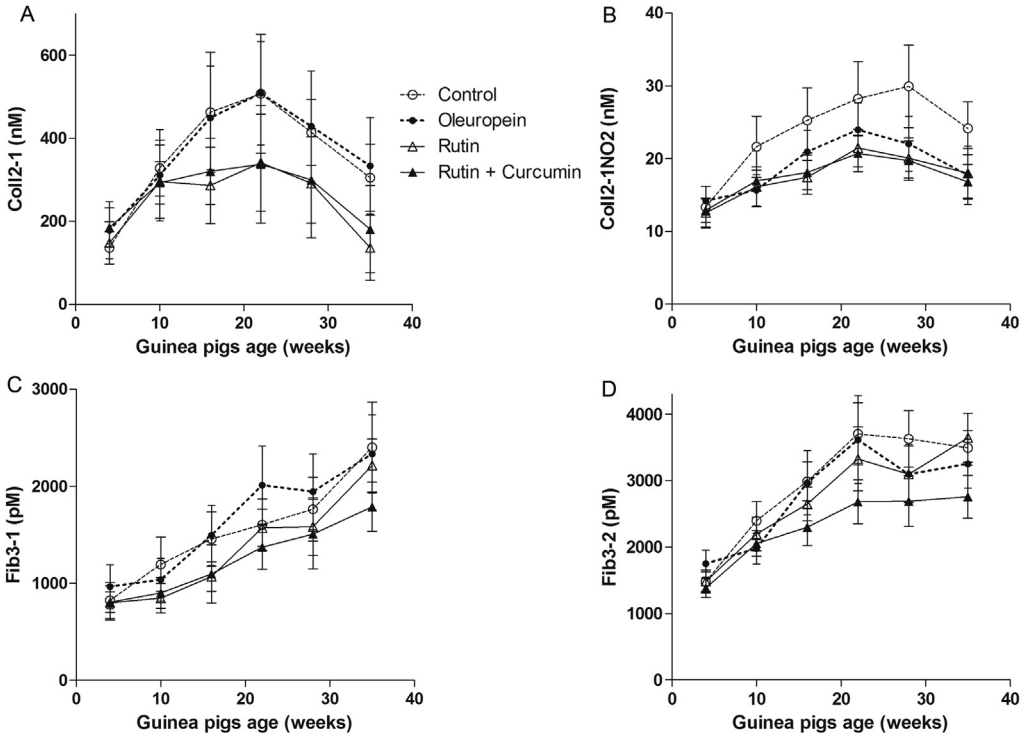

Fig. 4. (A) Coll2-1 (B) Coll2-1NO2 (C) Fib3-1 (D) Fib3-2 levels found in guinea pig sera. Means ± 95% CI.

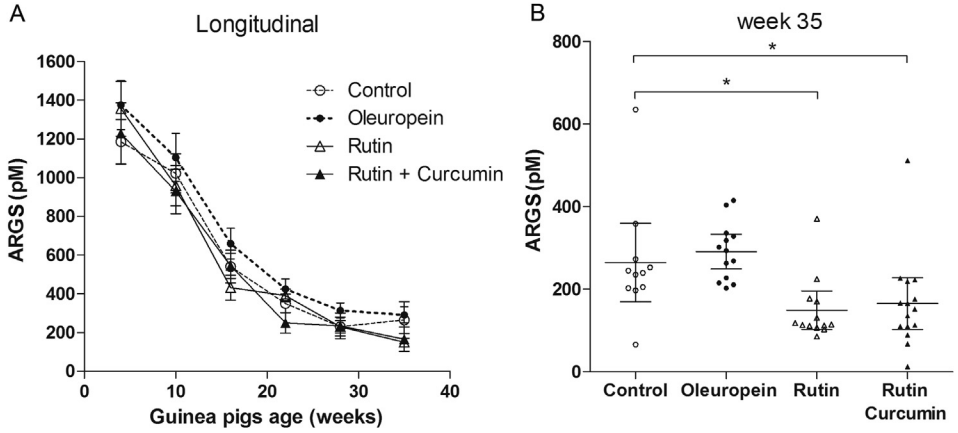

Fig. 5. ARGS levels found in guinea pig sera. (A) across time (B) at week 35. Means ± 95% CI, * = $P < 0.05$ one-way ANOVA with Dunnet's post-test.

between week 4 and 10. Finally, our conclusions would be strengthened by comparing Dunkin–Hartley to Strain 13 and Bristol strain 2 guinea pigs. In these guinea pigs, OA lesions are delayed and they could be used as comparator, even if there are not OA free at 35 weeks[2,39].

In conclusion, oleuropein and rutin induced interesting metabolic and structural effects on OA cartilage and synovium supporting their use in human trials. While the mixture of curcumin and rutin did not provide additive or synergistic effects compared to rutin alone on histological score, the combination of rutin and oleuropein might be worth investigating further due to their complementary mechanisms of action, protecting against pro-inflammatory and catabolic processes involved in development of OA. Coll2-1NO2 could be a good biomarker to monitor the protective effects of these compounds in guinea pig or human studies.

Author contribution

The authors' responsibilities were as follows—CS, MNH, EO, YH: designed the research and wrote the manuscript; CS, PD, FC, ST conducted the research; CS, AFD: performed statistical analyses; MNH, FM provided essential materials; CS: had primary responsibility for the final content of the manuscript; and all authors: read and approved the final manuscript.

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Competing interest statement

MN, FM and EO are employee of Nestle Research Center but have no conflict of interest to declare. CS, FC, AFD and PD have no conflict of interest. ST is employee of Artialis. YH is the founder of Artialis SA a spin-off of University of Liège. He has also received consulting fees from Tilman, Galapagos and BioIberica.

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