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ORIGINAL ARTICLE

The short-term effect of glucosamine-sulfate, nonanimal chondroitin-sulfate, and S-adenosylmethionine combination on ultrasonography findings, inflammation, pain, and functionality in patients with knee osteoarthritis: A pilot, double-blind, randomized, placebo-controlled clinical trial

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ABSTRACT

Objectives: This study aimed to investigate the efficacy of glucosamine-sulfate (GS), nonanimal chondroitin-sulfate (naCS), and S-adenosylmethionine (SAMe) combination on ultrasound findings, inflammation, pain, and functionality in knee osteoarthritis.

Patients and methods: In the prospective, randomized, double-blind, placebo-controlled pilot study conducted between August 2019 and November 2019, 120 participants (28 males, 92 females; mean age: 66.4±7.9 years; range, 42.4 to 74.5 years) were randomized at a 1:1:1 ratio to the placebo group, the first experimental group (a combination of GS, naCS, and SAMe was administered to the experimental groups. The first experimental group received 375 mg of GS, 300 mg of naCS, and 100 mg of SAMe, whereas the second experimental group received 750 mg of GS, 600 mg of naCS, and 200 mg of SAMe). Laboratory (erythrocyte sedimentation rate, C-reactive protein, tumor necrosis factor alpha, interleukin [IL]-1β, IL-6, IL-17), clinical (Visual Analog Scale [VAS], short form health survey [SF-36], the Western Ontario and McMaster Universities Arthritis Index [WOMAC], and the Tegner Lysholm Knee Scoring Scale [TLKS]), and musculoskeletal ultrasound (MSUS) assessments were performed at baseline and after three and six months.

Results: A minor increase was observed in the second experimental group after six months using ultrasonography to evaluate articular cartilage thickness ($p<0.05$). The investigational product's superiority in reducing osteoarthritis ultrasonographic findings was not proven. A moderately negative association was found between cartilage thickness and VAS scores at baseline ($\rho=-0.36$, $p<0.01$), while the presence of massive osteophytes on MSUS showed a low to moderate association with all clinical outcomes. There was no difference in the delta changes between groups for the VAS, TLKS, WOMAC, and SF-36. The only serum inflammatory marker outside the reference range was IL-1β, but no significant changes were observed after six months.

Conclusion: According to the results of our investigation, treatment for knee osteoarthritis should be evaluated using more objective outcomes. The most important conclusion of our study is that IP may result in a slight increase in articular cartilage thickness, which was associated with a decrease in pain intensity at baseline. Clarification of the potential influence of this combination on radiographic progression and laboratory markers of inflammation requires further exploration.

Keywords: Cartilage, chondroitin-sulfate, glucosamine-sulfate, knee osteoarthritis, s-adenosylmethionine.

Osteoarthritis (OA) is a progressive low-grade inflammatory and degenerative joint disease and the most ubiquitous type of arthritis in the human population. It is also the major cause of chronic musculoskeletal pain, joint instability, and decreased mobility in older adults worldwide.[1] Pathoanatomical changes, which can be seen in knee osteoarthritis (KOA), include

progressive destruction and consecutive loss of articular cartilage, subchondral bone remodeling, osteophyte formation, and low-grade synovium inflammation followed by synovial fluid effusion, as well as joint capsule hypertrophy and degeneration of ligaments of the knee.[2] These implicate that other components of the joint must be assessed as active contributors to disease progression and possible therapeutic targets, even though articular cartilage is the main focus of KOA studies.

Magnetic resonance imaging (MRI) is considered to be the gold standard for evaluating knee cartilage thickness. Nevertheless, ultrasonography (US) has arisen as a feasible alternative imaging technique for evaluating patients with painful joints.[3] Furthermore, it has been demonstrated that US enables similar sensitivity in the assessment of cartilage thickness in comparison to MRI.[4,5] Comparing US to MRI, it also provides trustworthy comprehensive imaging of soft tissues, such as cartilage, meniscus, ligament/tendon, synovium, and fluid collections.[6,7]

The treatment of KOA includes various nonpharmaceutical and pharmaceutical interventions.[8] The baseline pharmaceutical therapy includes the usage of symptomatic slow-acting drugs for osteoarthritis (SYSADOAs) for symptomatic KOA,[9] while others recommend topical and oral nonsteroidal anti-inflammatory drugs (NSAIDs) as the first line.[10,11] Regarding the other guidelines and recommendations, usage of SYSADOAs is still under debate, with insufficient evidence of efficacy, rather than their safety, for their usage. However, although fast-acting, NSAIDs do not have any disease-modifying potential (concept of disease-modifying osteoarthritis drugs [DMOADs]), often accompanied by side and toxic effects. Therefore, further clinical trials should be directed towards SYSADOAs since a substantial number of studies have suggested their anti-inflammatory effect and positive influence on cartilage thickness, accompanied by a low incidence of side effects.[12]

The most well-known and used SYSADOAs, glucosamine-sulfate (GS) and chondroitin-sulfate (CS), are considered to be components of the extracellular matrix of the articular cartilage and have been used for the prevention and management of osteoarthritis for more than 40 years.[13] S-adenosylmethionine (SAMe) is an activated form of methionine and a methyl group donor that plays key role in different biochemical reactions, gene expression, and protein synthesis.[14] New insights in pathogenesis of KOA clearly suggest a crucial role of proinflammatory cytokines such as tumor necrosis factor alpha (TNF)-α, interleukin (IL)-1, IL-6, and IL-17 at the intra-articular level,[15] with serum concentrations of TNF-α and IL-6 supporting the same.[16,17] We hypothesize that using SAMe could reduce oxidative stress and have an anti-inflammatory effect by suppressing cytokine secretion.

A study evaluating the efficacy of the combination of these three nutraceuticals in KOA has not been published yet. Moreover, there are a lack of studies using musculoskeletal US assessment as an outcome measure. This study aimed to investigate the efficacy of a fixed oral formulation of GS, nonanimal CS (naCS), and SAMe on cartilage thickness and other musculoskeletal US findings, inflammation, pain intensity, and functionality in patients with KOA.

PATIENTS AND METHODS

The prospective, randomized, double-blind, placebo-controlled pilot study was performed in a single center between August 2019 and November 2019. A total of 476 consecutive participants were recruited at the Institute of Rheumatology, University of Belgrade. During the screening period, participants' clinical or radiological diagnosis of symptomatic KOA was confirmed based on the American College of Rheumatology definition. Eligibility criteria for enrollment in the study were as follows: aged between 40 and 75, body mass index >20 kg/m^2 and <35 kg/m^2, Kellgren and Lawrence scale Grade 1-3, NSAID nonresponders, and pain intensity of 30-80 mm on the Visual Analog Scale (VAS). Participants were obligated to quit analgesics (both NSAIDs and non-NSAIDs) two weeks before enrollment and afterward, and with any type of physical rehabilitation (except for kinesiotherapy performed at home). Only acetaminophen up to 3 g/daily was permitted as rescue medication, except for 48 h before the scheduled visit and evaluation. Exclusion

criteria for the participants were as follows: Kellgren and Lawrence Grade 4, diagnosis with secondary KOA (metabolic or traumatic) and other knee disorders due to systemic or local inflammatory diseases (rheumatoid arthritis or other inflammatory arthropathies, chronic connective tissue diseases, history of septic arthritis), any prior or planned joint replacement or planned surgical intervention in general, fibromyalgia, and intraarticular or systemic usage of corticosteroid drugs one month before and during the trial. Participants suffering from a chronic heart or kidney disease, or another condition that could impair assessments, such as various neurological and psychiatric disorders, and patients with known allergy/hypersensitivity to any investigational product (IP) ingredient were not included. Finally, 240 knees of 120 participants (28 males, 92 females; mean age: 66.4±7.9 years; range, 42.4 to 74.5 years) were recruited for further clinical, laboratory, and imaging assessments. The patients were randomized at a 1:1:1 ratio and assigned to the placebo group, taking two capsules of placebo twice daily, the first experimental group, taking one capsule of IP and one capsule of placebo twice daily (up to 375 mg of GS, 300 mg of naCS, and 100 mg of SAMe), and the second experimental group, taking two capsules of IP twice daily (up to 750 mg of GS, 600 mg of naCS, and 200 mg of SAMe). Laboratory, clinical, and imaging assessments were performed at Visit 1, Visit 2 (at the third month of follow-up), and Visit 3 (at the sixth month of follow-up). The participant flow diagram is shown in Figure 1.

The primary endpoints were decrease in reported pain intensity after six months and increase or no changes in articular cartilage thickness after six months assessed by US.

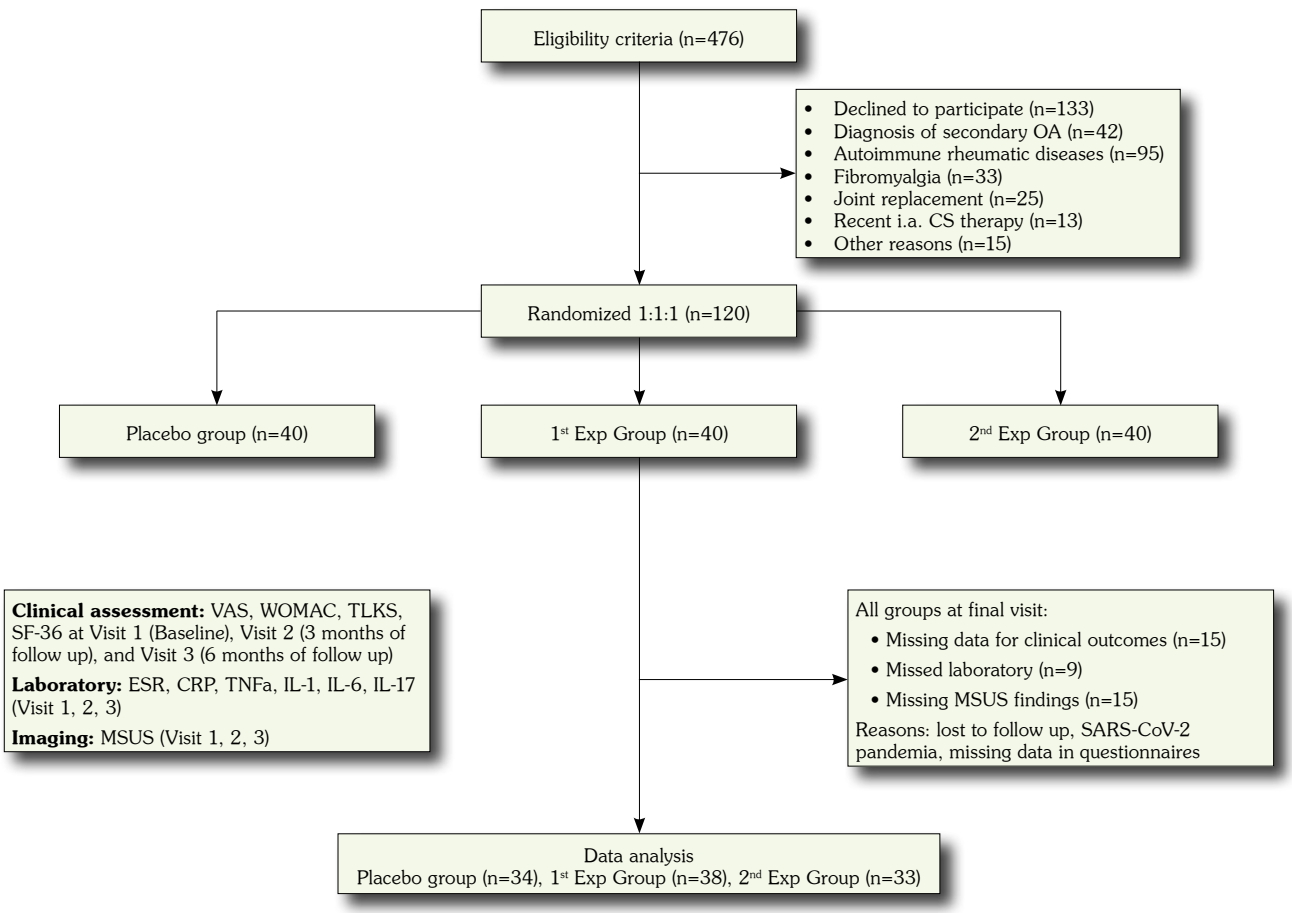


Figure 1. CONSORT flowchart.

OA: Osteoarthritis; VAS: Visual Analog Scale; WOMAC: Western Ontario and McMaster Universities Arthritis Index; TLKS: Tegner Lysholm Knee Scoring Scale; SF-36: 36-Item Short Form Survey Health Survey; MSUS: Musculoskeletal ultrasound; ESR: Erythrocyte sedimentation rate; CRP: C-reactive protein; TNF-α: Tumor necrosis factor alpha; IL: Interleukin; SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2.

The secondary endpoints were improvement in functionality (through total Western Ontario and McMaster Universities Arthritis Index [WOMAC] index and the Tegner Lysholm Knee Scoring Scale [TLKS] scale) and quality of life (through the Short Form Health Survey [SF-36]) after six months, decrease in the value of laboratory inflammatory markers after six months, and decrease or no changes in presence of ultrasonographic KOA-related findings after six months.

Description of the investigational product

The IP was manufactured and distributed in one capsule as a fixed-dose combination (Exedol®, 400 mg; AbelaPharm, Belgrade, Serbia) containing 187.5 mg of GS, 150 mg of naCS, 50 mg of SAMe, 12.0 mg of vitamin C, 0.75 mg of manganese-gluconate, 2.5 µg of cholecalciferol, and other excipients: scrub, talc, magnesium stearate, and silicon dioxide. The placebo was manufactured and distributed in one 400 mg capsule of the same shape, flavor, and color as IP, containing the same excipients as IP without active substances. After baseline assessment completion and depending on their randomization group, participants were supplied with IP/placebo and instructed to take two capsules twice per day for the next three months with consecutive resupply. Remaining unused capsules of each participant were collected at Visit 2 and Visit 3 (end of the trial). If a compliance rate of ≥80% was achieved, patient data were included in the analysis.

Clinical assessment questionnaires

The following questionnaires were used to estimate functional status, the severity of the symptoms, pain level, and health-related quality of life of patients.

The level of pain was recorded on the VAS by making a mark on a 100-mm line, and it was expressed in millimeters, where higher values indicated greater pain.

The TLKS was used for functionality assessment of KOA patients. They were expected to answer multiple-choice questions on eight items: pain, swelling, instability, locking, limping, stair climbing, support, and squatting. The total score ranges from 0 to 100, where higher scores indicate fewer symptoms and better functionality.[18]

WOMAC scale is a commonly used instrument in the evaluation of different treatment protocols in KOA patients. The WOMAC consists of three subscales (pain, stiffness, and physical function), with 24 items that need to be answered on a 5-point Likert scale. A higher score in certain subscales indicates greater pain and stiffness and worse physical function.[18]

The SF-36 is a widely used tool for health-related quality of life assessment, and it covers eight general domains. Two composite scores are available to summarize these domains: physical composite score (PCS) and mental composite score (MCS), followed by the total SF-36 score. All these scores are presented on a 0-100 scale, with higher scores reflecting better health-related quality of life.[19]

Imaging assessments

Musculoskeletal US was performed by one experienced ultrasonographer (10 years of experience). Both knees were examined with an EsaoteMyLab 50 machine (Esaote S.p.A, Genova, Italy) using a 12 MHz linear transducer. The participants were in a supine position with the knee in maximal flexion for femoral articular thickness assessment. The thickness was measured with a suprapatellar transverse scan at mid-points of the lateral condyle (LC), medial condyle (MC), and intercondylar notch (ICN) three times, and the arithmetic mean was taken. Three image acquisitions were performed for each patient on the scheduled visit. After all patients performed their visit, the ultrasonographer measured their cartilage thickness on acquired images in blocks of 10 patients (30 images) randomly. All other OA-related ultrasonographic findings were assessed after six months and scored with a specific dichotomous scale.[20] The presence of osteophytes was recorded in the supine position at both knees in complete extension and reported as not present/discrete or massive. A supine position with the knee in 30° flexion was used to identify the synovial fluid in the suprapatellar recess using a longitudinal scan, and it was reported as absent or present; the same position and scale were used for synovial hypertrophy (SH) assessment. The cut-off value for effusion and SH presence was >4 mm in depth on greyscale.[21] The presence of a popliteal cyst was assessed in the medial popliteal space with

patients in a prone position using a longitudinal and transverse scan. According to a paper published by the OMERACT (Outcome Measures in Rheumatology) task force, US assessment of structural and inflammatory changes on knees affected by OA is reliable.[22]

Laboratory assessment

Participants were obliged to be in a fasting state before blood drawing (09:00 AM). Blood samples were obtained, centrifuged, and stored at –70°C pending the analysis. Erythrocyte sedimentation rate (ESR) was manually measured (BD Seditainer; BD Vacutainer Systems, Plymouth, UK), and C-reactive protein (CRP) levels were measured with an automatic biochemical analyzer (Mindray BS-600; Mindray Bio-Medical Electronics Co., Ltd., Shenzhen, China). TNF-α, IL-1β, IL-6, and IL-17 were assayed with an enzyme-linked immunosorbent assay (ELISA) kit (Lot. P209961, Lot. P213811, Lot. P208259, and Lot. P195763, respectively; R&D Systems, Minneapolis, MN, USA). Results were obtained using a microplate reader (ProMedia, Kikinda, Serbia) and an online software (MyAssays, https://www.myassays.com).

Safety assessments

At the subsequent visits, all participants had their safety evaluated. At each follow-up visit, the investigator reviewed and recorded any adverse events (from moderate to severe). They were specifically questioned about headaches and gastrointestinal side effects like nausea, vomiting, diarrhea, and constipation. An adverse event was noted if it occurred twice for three consecutive days between two follow-up visits.

Sample size calculation and randomization method

The sample size was based on literature findings indicating that the mean VAS change in the experimental group will be 4±7.2 units higher compared to placebo. A sample size of 80 in the experimental group and 40 in the control group achieves 80% to detect a significant difference between groups, using an alpha of 0.05. Regarding other primary endpoints, we could not calculate sample size for change in cartilage thickness due to the lack of similar studies.

The randomization list was created by the study statistician using the complete (simple) randomization algorithm with three groups, equal sizes, and 1000 maximum iterations. The randomization list was created in R statistical software, randomizeR package version 1.4.2 (https://www.rdocumentation.org/packages/randomizeR/versions/1.4.2). In parallel, 120 randomly generated sequences of letters and digits were generated and sent to the drug production facilities. An independent researcher connected the randomization list and sequences for drug labeling. Drug packages were labeled based on the randomization list sequence by assigning the generated sequence of letters and numbers. In this manner, double blinding was achieved, and the list of codes was saved in the production facility. Each box with study drugs was marked with a six-symbol (letters and numbers) code and transported to the clinic. In the clinic, drug boxes were assigned to the patients according to the randomization number. The allocation of the patients remained unknown until the completion of the study when unblinding was performed, and each patient was assigned to the dedicated study group.

Statistical analysis

All data were analyzed using R version 4.0.2 (R Foundation for Statistical Computing, Vienna, Austria; https://www.R-project.org/). Results are presented as frequency (%), mean ± standard deviation, or median (interquartile range) depending on data type and distribution. Groups were compared using parametric (analysis of variance) and nonparametric tests (Kruskall-Wallis for continuous data and Pearson's chi-square test for nominal data). A Bonferroni-corrected p-value for all multiple comparisons was performed, and $p \leq 0.017$ was considered significant (when p was <0.05). The possible correlations between outcomes were assessed using Spearman's rank correlation coefficient. Blinded test-retest reliability for US measurement was performed using the intraclass correlation coefficient (ICC). Due to the COVID-19 (coronavirus disease 2019) outbreak during the study, imputation methods were applied to preserve the maximum number of available data. The "last observation caried forward" method was applied, but only in situations where one measurement was missing. In situations where two of three measurements were missing, no imputation was performed. Proportion of missing data for outcomes patients' reqruitment were

performed in September/October 2019. 11% of the data for the ultrasound assessment was missing for the 6th month visit due to the COVID 19 outbreak in March in Serbia. A *p*-value <0.05 was considered statistically significant.

RESULTS

There was no difference across the groups regarding age, sex, body mass index, and presence of other chronic concomitant diseases at baseline.

Clinical outcomes

Within-group mean changes of each outcome are shown in Appendix. Baseline measurements demonstrated no difference between groups (p>0.05). VAS values are shown in Table 1. There was a decrease in pain intensity in all three groups, with the most prominent changes in the placebo group after three months (p>0.05). Within-group mean changes in TLKS are shown in Appendix 1. There was an increase in total score in all three groups, without difference between groups (p>0.05). Changes in WOMAC score are shown in Appendix 2. There was a decrease in each WOMAC subscale and total score after three months. Regarding the pain subscale (A), the highest decrease was in the placebo group (-2.15±4.37), with same pattern in the function subscale (C) and the total score (D), suggesting a huge initial impact of placebo, despite no significant differences between groups after three and six months (p>0.05). Within-group mean changes for SF-36 are shown in Appendix 3. No difference between groups was noticed after three and six months. (p>0.05).

Laboratory markers of inflammation

Values of investigated markers of inflammation and their intragroup and intergroup differences are shown in Appendix 4. Regarding values of ESR, a reduction of 2 mm/h was observed in the first experimental group and second experimental group after six months (p>0.05). Baseline values of CRP were significantly different between groups, with higher, abnormal values in the first experimental group (cut-off: 5 mg/dL). After three months, CRP values in this group dropped (-0.3 mg/dL, 95% confidence interval -3 to 1.2). After six months, there was no difference between groups in the level of TNFα, IL-1β, IL-6, and IL-17 (p>0.05). It is important to note that only IL-1β was above the upper limit.

OA-related ultrasonographic characteristics

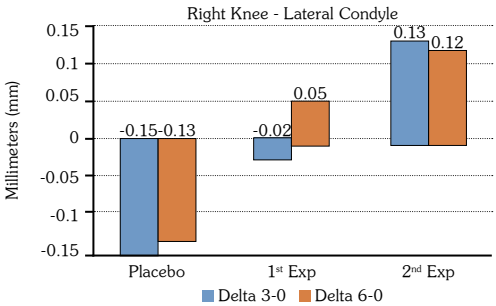
After six months, almost all patients in investigated groups had similar findings of SH compared to the baseline in the left knee (Appendix 5a). The highest variation in the presence of SH was found in the first and second experimental groups regarding the right knee. In 13% of patients in the first experimental group, SH disappeared, while in 12% of patients in the second experimental group, SH appeared (p>0.05). Regarding the presence of synovial fluid (Appendix 5b) in the left knee, about 90% of patients in the experimental groups had similar findings after six months compared to baseline. In 18% of patients in the placebo

Table 1. Visual Analog Scale scores

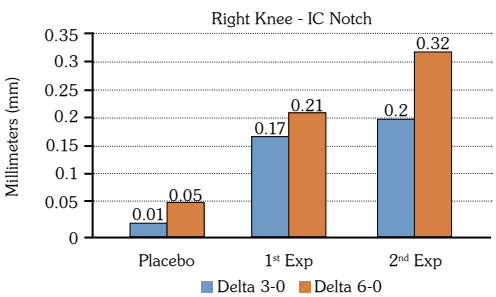
	Placebo (n=37)	1st Exp (n=39)	2nd Exp (n=36)	
VAS pain	Mean±SD	Mean±SD	Mean±SD	*p*
0 m	59.73±11.66	58.97±14.29	60.83±14.22	0.836
3 m	37.84±21.75	43.59±22.06	46.39±23.56	0.255
6 m	33.51±20.58	37.69±19.26	37.78±22.94	0.606
Δ 3-0	-21.89±22.09	-15.38±21.75	-14.44±22.1	0.288
Δ 6-0	-26.22±22.28	-21.28±22.73	-23.06±24.94	0.649

VAS: Visual Analog Scale; SD: Standard deviation; 0 m: Values at Baseline; 3 m: Values at three months; 6 m: Values after six months of supplementation, End of the study; Δ 3-0: Difference in values after three months of supplementation; Δ 6-0: Difference in values after 6 months of supplementation.

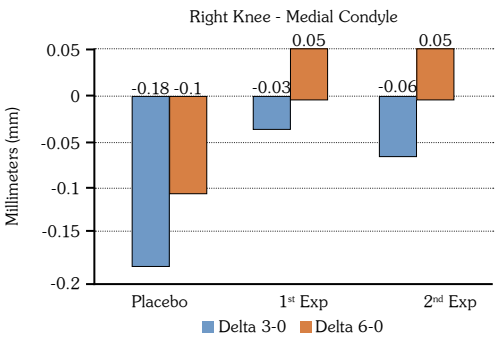
(a)



MSUS (mm)	*p* value	1st/Pcb *p* value	2nd/Pcb *p* value	1st/ 2nd *p* value
R LC Δ 3-0	0.075	0.283	0.022	0.211
R LC Δ 6-0	0.02	0.313	**0.009***	0.052

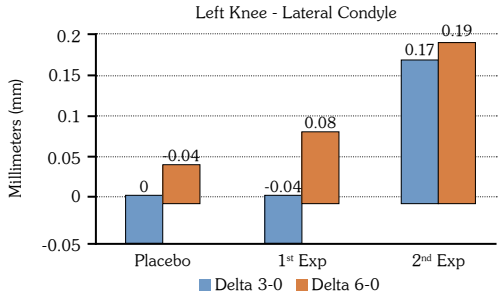


MSUS (mm)	*p* value	1st/Pcb *p* value	2nd/Pcb *p* value	1st/ 2nd *p* value
R ICN Δ 3-0	0.340	0.312	0.144	0.700
R ICN Δ 6-0	0.058	0.222	**0.017***	0.231

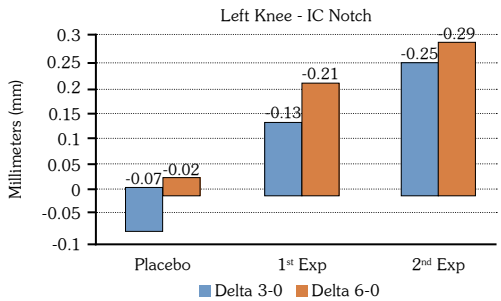


MSUS (mm)	*p* value	1st/Pcb *p* value	2nd/Pcb *p* value	1st/ 2nd *p* value
R MC Δ 3-0	0.466	0.23	0.414	0.755
R MC Δ 6-0	0.074	0.047	0.054	0.979

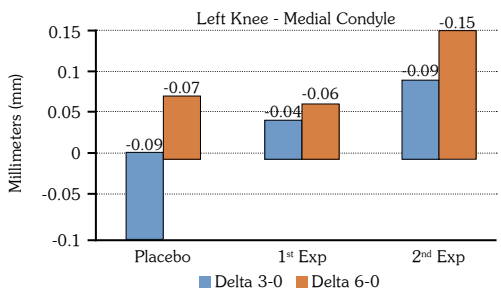
(b)



MSUS (mm)	*p* value	1st/Pcb *p* value	2nd/Pcb *p* value	1st/ 2nd *p* value
L LC Δ 3-0	0.208	0.896	0.153	0.092
L LC Δ 6-0	0.058	0.715	**0.056**	0.033



MSUS (mm)	*p* value	1st/Pcb *p* value	2nd/Pcb *p* value	1st/ 2nd *p* value
L ICN Δ 3-0	0.045	0.099	0.021	0.295
L ICN Δ 6-0	0.019	0.163	**0.006***	0.129



MSUS (mm)	*p* value	1st/Pcb *p* value	2nd/Pcb *p* value	1st/ 2nd *p* value
L MC Δ 3-0	0.317	0.274	0.155	0.631
L MC Δ 6-0	0.673	0.795	0.412	0.511

Figure 2. **(a)** Delta changes in the mean articular cartilage thickness (after 3 and 6 months) and differences between groups (right knee), * p≤0.017. **(b)** Delta changes in the mean articular cartilage thickness (after 3 and 6 months), and differences between groups in the left knee, * p≤0.017.

MSUS: Musculoskeletal ultrasound; R: Right knee; L: Left knee; LC: Lateral condyle; R ICN: Intercondylar notch; R MC: Medial condyle.

group, synovial fluid disappeared. Regarding the right knee, about 80% of patients in the investigated groups had similar findings after six months (p>0.05). After six months, almost all patients in the investigated groups had similar findings of popliteal cyst compared to baseline in both knees (Appendix 5c). Regarding the presence of osteophytes (Appendix 5d), 85% of patients in the investigated groups had similar findings in both knees after six months compared to baseline (p>0.05).

Articular cartilage thickness

Regarding test-retest reliability, the ICC was calculated using articular thickness measurements over the MC. An ICC of 0.936 showed good intrarater reliability (p<0.01). At the baseline and three and six months, there was no statistically significant difference between groups in cartilage thickness at all measured sites in both knees (p>0.05, Appendix 5e).

Regarding the right knee, there was a significant increase in the second experimental group compared to placebo in LC after six months (p=0.009). There was an increase in both experimental groups in ICN after six months, but the difference was significant only in the second experimental group compared to placebo (p=0.017). Regarding MC, thickness remained the same in both experimental groups and slightly decreased in the placebo group after six months, with a nonsignificant difference comparing both experimental groups to placebo (p=0.047 and p=0.054, respectively; Figure 2a).

Regarding the left knee, there was a nonsignificant increase in the LC in the second experimental group after six months compared to placebo and the first experimental group (p=0.033 and p=0.056, respectively). Regarding ICN, there was an increase in both experimental groups after three and six months, but it was significant only for the second experimental group compared to placebo after six months (p=0.006). Regarding MC, a slight increase occurred in all groups after six months (p>0.05, Figure 2b).

Correlations between ultrasonography findings, markers of inflammation, and clinical outcomes

Total cartilage thickness on both knees was shown to be negatively correlated with VAS scores at baseline in a statistically significant moderate manner (ρ=-0.36, p<0.01). The presence of osteophytes on the right or left knee was substantially correlated with VAS, TLKS, WOMAC subscales, SF-36 PCS, and SF-36 total (Table 2).

A low level of negative correlation between ESR, TLKS, and SF-36 was found at baseline (p<0.01 and p<0.05 respectively). Regarding proinflammatory cytokines, only a low level of positive correlation was observed between IL-17 and VAS (ρ=0.20, p<0.05, Table 3).

Table 2. Baseline correlations of ultrasonography findings and clinical outcomes

	VAS	TLKS	W pain	W stiffness	W function	W total	SF-36 PCS	SF-36 MCS	SF-36 total
Total CT	-0.36**	-0.05	-0.15	-0.18	-0.11	-0.15	0.08	0.10	0.09
R SH	-0.01	0.10	-0.16	-0.14	-0.20	-0.13	0.09	-0.02	0.03
R SF	-0.09	-0.03	-0.08	-0.12	0.02	-0.08	-0.04	-0.06	-0.05
R OP	0.21*	-0.35*	0.17	0.26*	0.25*	0.22	-0.16	-0.03	-0.08
R PC	0.16	-0.02	0.06	0.08	0.13	0.10	-0.17	-0.06	0.04
L SH	-0.12	-0.20	0.13	0.04	0.10	-0.06	0.14	-0.02	0.06
L SF	0.11	-0.01	-0.03	-0.01	-0.02	-0.19	0.04	-0.01	0.04
L OP	0.26*	-0.41**	0.24	0.26*	0.36*	0.23	-0.28*	-0.21	-0.31*
L PC	0.13	-0.08	0.15	0.02	0.01	0.07	-0.16	-0.11	-0.12

VAS: Visual Analog Scale of pain intensity; TLKS: The Tegner Lysholm Knee Scoring Scale; W: The Western Ontario and McMaster Universities Arthritis Index; SF-36: 36-Item Short Form Survey Health Survey; PCS: Physical composite score; MCS: Mental composite score; Total CT: Mean cartilage thickness on both knees, R: Right knee; L: Left knee; SH: Synovial hypertrophy; SF: Synovial fluid; OP: Osteophytes; PC: Popliteal cyst; * -<0.05; ** <0.01.

Table 3. Baseline correlations of markers of inflammation and clinical outcomes

	VAS	TLKS	W pain	W stiffness	W function	W total	SF-36 PCS	SF-36 MCS	SF-36 total
ESR	0.05	-0.30**	0.0	0.10	0.13	0.17	-0.24*	-0.20*	-0.22*
CRP	0.12	-0.12	0.17	0.14	0.11	0.14	-0.08	-0.09	-0.09
TNF-α	0.09	0.03	-0.09	-0.09	0.06	0.07	0.06	0.11	0.11
IL-1	0.11	0.10	-0.10	0.07	-0.02	-0.03	-0.05	0.00	-0.02
IL-6	-0.03	-0.04	0.11	0.12	0.11	0.12	-0.03	-0.10	-0.02
IL-17	0.20*	0.17	-0.04	-0.04	-0.06	-0.07	0.15	0.14	0.16

VAS: Visual Analog Scale of pain intensity; TLKS: The Tegner Lysholm Knee Scoring Scale; W: The Western Ontario and McMaster Universities Arthritis Index; SF-36: 36-Item Short Form Survey Health Survey; PCS: Physical composite score; MCS: Mental composite score; ESR: Erythrocyte sedimentation rate; CRP: C-reactive protein; TNF-α: Tumor necrosis factor alpha; IL: Interleukin; * -<0.05; ** <0.01.

Safety assessments

Nausea and dyspepsia were the most commonly reported adverse events (Appendix 6). However, there was no statistically significant difference between the groups.

DISCUSSION

The presented study was designed to access various clinical, laboratory, and imaging outcomes for one IP in patients with KOA. The combination of these three SYSADOAs has not yet been studied. As outcome measures, several KOA-related ultrasonographic features and serum inflammatory biomarkers were evaluated, as well as their correlations with clinical findings.

The results revealed a modest but considerable increase in cartilage thickness measured by musculoskeletal US after six months using a higher-dose GS, CS, and SAMe combination, suggesting a potential increase in articular cartilage volume. According to baseline correlations between musculoskeletal US findings and clinical outcomes, we identified a significant moderate negative correlation between articular cartilage thickness and the VAS score, which implies that patients report less pain as cartilage thickness increases. After six months, there is no proof to back up the IP's superiority in reducing different KOA-related ultrasonographic characteristics, including osteophytes, as their presence at baseline demonstrated substantial correlations with pain severity, functionality, and health-related quality of life.

We searched for studies evaluating the efficacy of the CS and GS alone or in combination on different clinical, laboratory, and US-related outcomes in patients with KOA due to the lack of studies evaluating SAMe.[23] We found more than 15 articles withmoderate to high scientific quality regarding our outcomes, published between 2000 and 2020, and possibly there are many more.[24] However, direct comparisons with these studies should be taken with caution due to differences in study protocol design, IP formulation, and IP doses. As an example, most of the studies used glucosamine-hydrochloride (GH) and N-acetyl glucosamine rather than GS. This difference is important, because following oral administration of a clinically recommended dose of GS, significantly higher synovial fluid concentrations of glucosamine are attained compared to an equivalent dose of GH.[25] Another difference is that few studies used CS of animal origin. In almost all previous studies, the dose of the study drug combination was 1500 mg of GH/GS daily and 1200 mg of CS daily, while the highest dose of IP in our study was twice lower (750 mg of GS and 600 mg CS) along with 200 mg of SAMe. There are several studies that used lower doses of CS.[26-29] The dosing regime was also different, with the study product being three times daily in other studies.

The studies have demonstrated that GS and CS can influence the cartilage thickness, at the same time promoting the synthesis of hyaluronic acid.[30] SAMe is known to indirectly protect

synovial cells by inducing glutathione peroxidase production and blocking the activity and synthesis of cartilage-degrading enzymes.[31] We hypothesized that a combination of GS, naCS, and SAMe could influence cartilage thickness and US indicators of inflammation in KOA considering all the previously mentioned beneficial mechanisms of these three nutraceuticals. There has not yet been a study that examines the CS+naGS+SAMe combination and uses musculoskeletal US to measure cartilage thickness and other KOA findings. There is MRI data suggesting that patients should take GS and CS for at least two years to see beneficial effects on cartilage loss,[32] but a few authors claim that beneficial effects of CS alone can be seen as early as six months.[33] There are few studies using musculoskeletal US as an outcome measure, and it suggests that CS alone could have an impact on the presence of synovitis in KOA,[34] while intra-articular application of the CS and hyaluronic acid combination showed promising but nonsignificant influence on synovial thickness, presence of joint effusion, and popliteal cyst.[35] In the study of Alayat et al.,[36] there was a significant decrease in synovial thickness in posttreatment period using a combination of GS/CS and Nd:YAG laser, while other studies evaluated changes in different US findings after the application of intra-articular steroids.[37,38] Our findings regarding the link between cartilage thickness, ultrasonographic findings, and pain intensity are consistent with those reported by Bernando-Bueno et al.,[39] who noticed that morphologic abnormalities on the US evaluation, such as cartilage thickness, were able to predict significant joint pain in KOA, whereas inflammatory changes in the same evaluation did not.

The reliability of the femoral articular thickness and the application value of US assessment in terms of diagnosis, follow-up, and prediction of KOA progression to total knee arthroplasty may be the study's weak point. As seen in two articles, this technique has been validated against X-ray, MRI, and arthroscopy,[40,41] and it is reliable and reproducible in both healthy people and normal to moderately damaged cartilage.[42-45] Moreover, novel publications introduced a semiautomated ultrasound technique for the evaluation of femoral articular thickness, which can be reliable even when used by unexperienced ultrasonographers.[46] The assessment of femoral articular thickness has been validated against pain scores, the WOMAC scale,[47,48] and muscle strength.[49] The medial compartment cartilage thickness loss was found to be associated with both concurrent and subsequent radiographic progression and with concurrent symptomatic progression,[50] with recent data suggesting that ultrasonographic examination of the knee and a machine learning method may provide added value to basic clinical and demographic descriptors in predicting total knee replacement in the future,[51] both contributing significantly to the process of establishing cartilage thickness as a biomarker of clinically relevant progression of knee OA.

Regarding pain intensity, our results suggest that placebo had the highest initial effect on pain reduction. A study published by Roman-Blas et al.[52] showed that a placebo was superior compared to a combination of GS and CS in pain reduction after six months in the experimental model. However, other studies showed that a combination of GH and CS, with or without other nutraceuticals, was superior in pain reduction compared to placebo after six months[53] or after 12 and 24 weeks.[54] Our results are in concordance with studies that showed that there is no difference in the glucosamine and chondroitin combination versus placebo after three months,[55] six months,[26,56] and up to one and two years.[27] Our results suggest that placebo also has a huge initial impact on total WOMAC score and related subscales. This is in concordance with the majority of previous studies, which suggest that a combination of glucosamine and chondroitin is not superior to placebo in total WOMAC score or related subscales after six months.[52,56-58] One study showed that the combination was superior to placebo in decreasing the total WOMAC score after three months.[55] Regarding the health-related quality of life, there was an increase in PCS and MCS in all groups, as well as in the total SF-36 score, suggesting a better quality of life. Our results are in concordance with the other two studies with follow-ups of two years[27] and four years.[59] Among laboratory markers of inflammation, only IL-1β was increased in sera of our patients,[15] which is a therapeutic target for KOA.[60] Our data suggest that a combination of

GS+CS+SAMe does not have an impact on ESR and CRP levels. The study of Rondanelli et al.[61] had similar outcomes as our study, evaluating the usage of the same dose (600 mg) of naCS alone, showing that CS was superior to placebo after 12 weeks in decreasing ESR and CRP. The same conclusions regarding CRP were presented by previously mentioned authors.[19] Moreover, Lugo et al.[56] showed that there are no differences in serum CRP and synovial fluid IL-6 in GS+CS compared to placebo, but another study claims that the CS+GS combination was superior to celecoxib in reducing IL-6.[62]

This study has several limitations. Most of our participants were female. Additionally, a considerable placebo effect occurred, particularly when evaluating clinical outcomes, even though no significance was noticed. Furthermore, interrater reliability was not tested since there was only one ultrasonographer who performed the measurements, and this study was performed in a single center. Moreover, US measurement of cartilage thickness is less specific than volumetric MRI. Among our primary endpoints, sample size was calculated only for change in VAS scores. This may be the reason why there was no difference in the levels of IL-1β. Due to the study design, we were not able to assess the potential level of SAMe efficacy on various outcomes independent of glucosamine and chondroitin. Our findings with this particular combination of GS+CS+SAMe cannot be extrapolated to other products currently on the market. Therefore, the collected data are insufficient for final reporting and can be presented as a preliminary report, but we encourage future work in this field.

In conclusion, the results of our study imply that much more objective measurements, such as the use of US and various laboratory indicators, might be employed as outcomes for comparing various SYSADOAs, given the significant influence of placebo on a range of clinical outcomes. The most significant finding from our study is that GS, naCS, and SAMe may lead to a slight increase in articular cartilage thickness, which in turn was linked to a lower pain intensity at baseline. It is necessary to perform a study comparing the combinations of GS+CS and GS+naCS+SAMe and placebo to assess the effects of SAMe on different outcomes independent of GS and naCS. Further research with a follow-up longer than six months is required for the clarification of the possible impact of this combination on radiographic progression, laboratory indicators of inflammation, and possible side-effects. The loss of cartilage and patient-reported outcomes may be impacted by other confounding factors during a longer follow-up period, such as the usage of NSAIDs and other concomitant medications, dietary habits, obesity, and other diseases; thus, appropriate study designs are required to overcome these limitations.

Ethics Committee Approval: The study protocol was approved by the Institute of Rheumatology (Belgrade, Serbia) Ethics Committee (date: 03.07.2019, no: 29/1-21). The study was conducted in accordance with the principles of the Declaration of Helsinki.

Patient Consent for Publication: Each participant who took part in the study signed a consent to publish form. They receive informations about the security protocol for protecting personal data.

Data Sharing Statement: The authors confirm that the data supporting the findings of this study are available within the article and/or its supplementary materials. Raw data were generated at Institute of Rheumatology. Derived data supporting the findings of this study are available from the corresponding author (G.R., Z.V.) on request.

Author Contributions: Idea/concept: G.R., Z.V.; Design: G.R., Z.V., S.M.; Control/supervision: G.R.; Data collection and/or processing: Z.V., N.S., S.J., L.K., I.S., B.S., T.Ž.R., V.B.; Analysis and/or interpretation: G.R., Z.V., S.P.D., I.S.; Literature review: G.R., Z.V., S.P.D.; Writing the article: G.R., Z.V., S.P.D., N.S., S.J., L.K., I.S.; Critical review: G.R., Z.V., S.P.D.; References and Funding: G. R., Z.V., S.M.; Materials: S.M.

Conflict of Interest: Company supplied study site with study drug and placebo for all patients. This is the first random-ized trial to evaluate efficacy of CS+naGS+SAMe. Company didn't interfere with study results and they agreed to accept all conclusions about efficacy of their product.

Dr Saša Janjić received fee as investigators for patient reqruitment and patients' study visits. Dr Ljiljana Kovačević received fee for laboratory analysis. Dr Ivan Soldatović received fee for protocol design and statistical analysis. Dr Zoran Veličković, Dr Slavica Pavlov Dolijanović, Dr Nikola Stojanović and Dr Goran Radu-nović declare no conflict of interest.

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Appendix 1. The Tegner Lysholm Knee Scoring scale values

	Placebo (n=35)	1st Exp (n=39)	2nd Exp (n=33)	
TLKS	Mean±SD	Mean±SD	Mean±SD	*p* value
0 m	46.69±20.27	43.9±17.38	37.39±20.87	0.136
3 m	55.97±21.74	55.95±19.48	51.42±23.98	0.608
6 m	61.83±23.4	59.00±21.17	55.33±26.65	0.534
Δ 3-0	9.29±18.86	12.05±21.65	14.03±28.36	0.697
Δ 6-0	15.14±20.56	15.10±24.34	17.94±26.96	0.855

TLKS: Tegner Lysholm Scoring Scale; SD: Standard deviation; 0 m: Values at Baseline; 3 m: Values at 3rd month; 6 m: Values after 6 months of supplementation, End of the study; Δ 3-0: Difference in values after 3 months of supplementation; Δ 6-0: Difference in values after 6 months of supplementation.

(a)

Appendix 2. WOMAC scores values

	Placebo (n=35)	1st Exp (n=38)	2nd Exp (n=34)	
WOMAC	Mean±SD	Mean±SD	Mean±SD	*p* value
Pain 0 m	11.06±4.84	10.05±3.38	11.26±3.64	0.252
Pain 3 m	8.91±4.28	8.32±3.74	10.35±3.95	0.098
Pain 6 m	8.39±4.86	8.03±4.04	9.00±4.42	0.618
Δ 3-0	-2.15±4.37	-1.74±2.95	-0.91±3.69	0.272
Δ 6-0	-2.67±5.24	-2.03±4.43	-2.26±4.96	0.869

(b)

	Placebo (n=34)	1st Exp (n=38)	2nd Exp (n=34)	
WOMAC	Mean±SD	Mean±SD	Mean±SD	*p* value
Stiffness 0 m	3.65±2.32	3.38±1.97	4.22±1.79	0.238
Stiffness 3 m	3.06±2.17	3.09±1.69	3.78±1.93	0.296
Stiffness 6 m	2.59±2.35	3.03±1.95	3.47±1.85	0.214
Δ 3-0	-0.59±2.31	-0.29±1.66	-0.44±2.20	0.643
Δ 6-0	-1.06±2.09	-0.35±1.92	-0.75±2.62	0.444

(c)

	Placebo (n=34)	1st Exp (n=38)	2nd Exp (n=34)	
WOMAC	Mean±SD	Mean±SD	Mean±SD	*p* value
Function 0 m	35.97±15.92	36.05±12.41	37.23±11.84	0.890
Function 3 m	26.56±14.88	29.58±10.50	34.23±13.62	0.111
Function 6 m	27.06±16.72	25.42±12.58	29.20±14.76	0.656
Δ 3-0	-9.41±13.74	-6.47±11.02	-3.00±12.26	0.302
Δ 6-0	-8.91±15.12	-10.63±11.91	-8.03±15.11	0.520

(d)

	Placebo (n=34)	1st Exp (n=38)	2nd Exp (n=34)	
WOMAC	Mean±SD	Mean±SD	Mean±SD	*p* value
Total 0 m	50.47±22.47	49.55±16.21	52.66±16.10	0.717
Total 3 m	38.38±20.43	41.13±14.14	48.37±18.57	0.100
Total 6 m	37.91±23.25	36.50±17.31	41.34±20.40	0.606
Δ 3-0	-12.09±18.82	-8.42±13.57	-4.29±16.70	0.379
Δ 6-0	-12.56±21.40	-13.05±16.55	-11.31±21.44	0.822

WOMAC: The Western Ontario and McMaster Universities Arthritis Index; SD: Standard deviation; 0 m: Values at Baseline; 3 m: Values at 3rd month; 6 m: Values after 6 months of supplementation, End of the study; Δ 3-0: Difference in values after 3 months of supplementation; Δ 6-0: Difference in values after 6 months of supplementation.

Appendix 3. The Short Form 36 Health Survey (SF-36)

	Placebo (n=35)	1st Exp (n=36)	2nd Exp (n=35)	
SF-36	Mean±SD	Mean±SD	Mean±SD	*p* value
PCS 0 m	36.68±15.72	35.51±15.01	35.97±14.77	0.950
PCS 3 m	46.29±19.68	42.06±18.37	40.86±18.73	0.460
PCS 6 m	50.18±21.16	44.8±19.06	48.11±20.3	0.537
Δ 3-0	9.62±19.53	6.54±17.59	4.89±18.91	0.568
Δ 6-0	13.50±20.65	9.29±17.54	12.14±22.75	0.682
MCS 0 m	49.24±19.00	45.06±17.78	51.34±17.59	0.340
MCS 3 m	54.53±18.87	51.74±20.13	51.49±20.54	0.781
MCS 6 m	60.15±20.09	54.69±19.92	56.4±18.44	0.496
Δ 3-0	5.29±18.75	6.69±18.02	0.14±23.4	0.366
Δ 6-0	10.91±20.51	9.63±21.78	5.06±26.21	0.539
Total 0 m	42.79±17.46	38.51±16.11	42.8±16.26	0.536
Total 3 m	51.32±19.47	47.49±19.41	46.51±19.16	0.560
Total 6 m	56.03±21.25	49.86±20.46	53.00±18.92	0.523
Δ 3-0	8.53±18.98	8.97±18.11	3.71±21.61	0.481
Δ 6-0	13.24±21.13	11.34±20.13	10.2±24.91	0.849

PCS: Physical Composite Score; MCS: Mental Composite Score; SD: Standard deviation; 0 m: Values at Baseline; 3 m: Values at 3rd month; 6 m: Values after 6 months of supplementation, End of the study; Δ 3-0: Difference in values after 3 months of supplementation; Δ 6-0: Difference in values after 6 months of supplementation.

(a)

Appendix 4. Values of laboratory markers of inflammation

	Placebo (n=35)	1st Exp (n=39)	2nd Exp (n=37)	
Laboratory	Median (Min-Max)	Median (Min-Max)	Median (Min-Max)	*p* value
ESR 0 m	16 (10-20)	18 (10-26)	18 (12-22)	0.564
ESR 3 m	16 (8-24)	14 (10-24)	14 (8-22)	0.889
ESR 6 m	18 (10-24)	18 (10-24)	16 (10-22)	0.808
Δ 3-0	0 (-4-2)	-1 (-6-2)	-2 (-4-2)	0.516
Δ 6-0	0 (-4-4)	-2 (-6-2)	-2 (-4-2)	0.521
CRP 0 m	3.2 (2.1-4.7)	6.65 (2.4-11.1)	4.4 (2.5-7.4)	0.023
CRP 3 m	3.9 (2.9-6.5)	5.55 (3.6-7.9)	4.1 (2.9-5.7)	0.055
CRP 6 m	3.9 (3-6.1)	5.55 (3.6-8.6)	4.3 (3.1-7)	0.169
Δ 3-0	1.1 (-0.3-1.9)	-0.3 (-3-1.2)	0.4 (-1.6-1.4)	0.059
Δ 6-0	0.9 (-0.2-2.1)	0.2 (-3-2.1)	0.3 (-1.6-2.2)	0.364
TNF 0 m	2.61 (0.97-5.76)	1.34 (0.92-3.48)	3.18 (1-6.62)	0.097
TNF 3 m	4.6 (1.08-7.59)	2.98 (1.1-6.83)	5.55 (2.19-8.7)	0.372
TNF 6 m	2.88 (1-6.08)	1.9 (1-3.68)	2.8 (0.95-4.4)	0.397
Δ 3-0	1.12 (0.05-2.41)	1 (0.05-2.7)	1.04 (0.08-2.65)	0.854
Δ 6-0	0.05 (-1.48-1.42)	0.02 (-1.18-1.01)	-0.1 (-1.77-0.6)	0.292

(b)

	Placebo (n=35)	1st Exp (n=39)	2nd Exp (n=37)	
Laboratory	Median (Min-Max)	Median (Min-Max)	Median (Min-Max)	*p* value
IL-1 0 m	3.65 (3.18-4.91)	4.44 (3.75-5.45)	3.94 (3.34-5.32)	0.223
IL-1 3 m	4.09 (3.53-5.32)	4.88 (3.97-6.24)	4.31 (3.56-5.71)	0.179
IL-1 6 m	3.9 (3.34-5.32)	4.16 (3.31-5.29)	3.94 (3.18-5.17)	0.726
Δ 3-0	0.35 (0.07-0.66)	0.35 (-0.13-0.98)	0.22 (0-0.7)	0.629
Δ 6-0	0.03 (-0.38-0.54)	-0.29 (-0.79-0.35)	-0.19 (-0.73-0.76)	0.404
IL-6 0 m	4.02 (3.28-4.37)	3.99 (2.84-4.99)	4.22 (2.78-6)	0.798
IL-6 3 m	4.05 (3.26-5.16)	4.72 (3.55-5.75)	4.28 (3.61-5.69)	0.378
IL-6 6 m	3.9 (1.94-4.84)	4.22 (2.2-5.34)	4.19 (3.58-5.15)	0.433
Δ 3-0	0.28 (-0.12-0.56)	0.56 (0.06-1.12)	0.41 (0.09-0.68)	0.072
Δ 6-0	-0.33 (-1.29-0.56)	-0.06 (-1.01-0.56)	0.15 (-0.76-0.9)	0.674
IL-17 0 m	7.99 (6.91-10.78)	7.99 (5.94-11.15)	7.27 (6.06-10.42)	0.719
IL-17 3 m	9.69 (7.75-12.1)	9.21 (8.12-12.24)	9.94 (7.88-13.57)	0.890
IL-17 6 m	9.69 (7.75-13.93)	9.57 (8.24-12.6)	10.29 (8.24-14.79)	0.742
Δ 3-0	0.97 (0.11-2.76)	1.81 (-0.36-3.39)	1.81 (0.73-3.39)	0.327
Δ 6-0	0.73 (-0.12-3.76)	1.45 (-1.09-3.39)	2.3 (0.73-5.09)	0.257

ESR: Estimated Sedimentation Rate; CRP: C-reactive protein; TNF: Tumor necrosis factor; IL: Interleukin; 0 m: Values at Baseline; 3 m: Values at 3rd month; 6 m: Values after 6 months of supplementation, End of the study; Δ 3-0: Difference in values after 3 months of supplementation; Δ 6-0: Difference in values after 6 months of supplementation.

Appendix 5a. Assessment of ultrasonographic characteristics - synovial hypertrophy

		Placebo (n=34)		1st Exp (n=38)		2nd Exp (n=33)		
MSUS		n	%	n	%	n	%	*p*
Left SH 0 m	Absent	26	76.5	30	78.9	26	78.8	0.787
	Present	8	33.5	8	21.1	7	21.2	
Left SH 6 m	Absent	29	85.2	32	84.2	27	81.8	1.000
	Present	5	14.8	6	15.8	6	19.2	
Left Δ SH	-1	3	9.8	3	7.9	2	6.1	1.000
	0	31	91.2	34	89.5	30	90.9	
	1	0	0.0	1	2.6	1	3.0	
Right SH 0 m	Absent	28	82.3	32	84.2	28	84.8	0.963
	Present	6	17.7	6	15.8	5	15.2	
Right SH 6 m	Absent	30	88.2	34	89.5	25	75.7	0.331
	Present	4	11.8	4	10.5	8	19.2	
Right Δ SH	-1	2	5.9	5	13.1	1	3.1	0.247
	0	32	94.1	30	78.9	28	84.8	
	1	0	0.0	3	8.0	4	12.1	

MSUS: Musculoskeletal ultrasound; SH: Synovial hypertrophy; 0 m: Baseline; 6 m: End of study; Δ SH: Change in presence of synovial hypertrophy after 6 months; -1: Synovial hypertrophy disappeared; 0: No change; 1: Synovial hypertrophy appeared.

Appendix 5b. Assessment of ultrasonographic characteristics - synovial fluid/effusion

		Placebo (n=34)		1st Exp (n=38)		2nd Exp (n=33)		
MSUS		n	%	n	%	n	%	*p*
Left SF 0 m	Absent	24	70.6	32	84.2	28	84.8	0.477
	Present	10	29.4	6	15.8	5	15.2	
Left SF 6 m	Absent	30	88.2	36	94.7	29	87.9	0.732
	Present	4	11.8	2	5.3	4	12.1	
Left Δ SF	-1	6	17.7	4	10.5	2	6.1	0.632
	0	28	82.3	34	89.5	30	90.9	
	1	0	0.0	0	0.0	1	3.0	
Right SF 0 m	Absent	25	73.5	26	68.4	21	63.6	0.774
	Present	9	16.5	12	31.6	12	26.4	
Right SF 6 m	Absent	30	88.2	32	84.2	23	69.7	0.453
	Present	4	11.8	6	15.8	10	30.3	
Right Δ SF	-1	6	17.6	7	18.4	4	12.1	0.806
	0	27	79.4	30	78.9	27	81.8	
	1	1	3.0	1	2.7	2	6.1	

MSUS: Musculoskeletal ultrasound; SF: Synovial fluid; 0 m: Baseline; 6 m: End of study; Δ SF: Change in presence of synovial fluid after 6 months; -1: Synovial fluid disappeared; 0: No change; 1: Synovial fluid appeared.

Appendix 5c. Assessment of ultrasonographic characteristics - popliteal cyst

MSUS		Placebo (n=34)		1st Exp (n=38)		2nd Exp (n=33)		p
		n	%	n	%	n	%	
Left PC 0 m	Absent	30	88.2	32	85.8	28	84.8	0.770
	Present	4	11.8	6	14.2	5	15.2	
Left PC 6 m	Absent	31	91.2	35	92.1	28	84.8	0.597
	Present	3	8.8	3	7.9	5	15.2	
Left Δ PC	-1	2	5.9	3	7.9	1	3.1	0.815
	0	31	91.2	35	92.1	31	93.9	
	1	1	2.9	0	0.0	1	3.1	
Right PC 0 m	Absent	29	85.3	31	81.6	29	87.9	0.636
	Present	5	14.7	7	18.4	4	12.1	
Right PC 6 m	Absent	31	91.2	35	92.1	27	81.8	0.723
	Present	3	8.8	3	7.9	6	17.2	
Right Δ PC	-1	3	8.8	5	13.1	1	3.0	0.480
	0	29	85.3	32	85.8	29	87.9	
		1	5.9	1	1.1	3	9.1	

MSUS: Musculoskeletal ultrasound; PC: Popliteal cyst; 0 m: Baseline; 6 m: End of study; Δ PC: Change in presence of popliteal cyst after 6 months; -1: Popliteal cyst disappeared; 0: No change; 1: Popliteal cyst appeared.

Appendix 5d. Assessment of ultrasonographic characteristics - osteophytes

MSUS		Placebo (n=34)		1st Exp (n=38)		2nd Exp (n=33)		p
		n	%	n	%	n	%	
Left OP 0 m	Absent	22	64.7	22	57.9	17	51.5	0.470
	Present	12	35.3	16	42.1	16	48.5	
Left OP 6 m	Absent	23	67.6	23	60.5	19	57.6	0.578
	Present	11	32.4	15	29.5	14	42.3	
Left Δ OP	-1	4	11.8	2	5.3	4	12.1	0.665
	0	27	79.4	35	92.1	27	81.8	
	1	3	8.8	1	2.6	2	6.1	
Right OP 0 m	Absent	19	55.9	17	44.7	18	54.5	0.627
	Present	15	44.1	21	55.3	15	45.5	
Right OP 6 m	Absent	20	58.8	17	44.7	20	60.6	0.599
	Present	14	41.2	21	55.3	13	29.4	
Right Δ OP	-1	3	8.8	2	5.3	4	12.1	0.822
	0	29	85.3	34	89.4	27	81.8	
		2	5.9	2	5.3	2	6.1	

MSUS: Musculoskeletal ultrasound; OP: Osteophytes; 0 m: Baseline; 6 m: End of study; Δ OP: Change in presence of osteophytes after 6 months; -1: Osteophytes became less prominent; 0: No change; 1: Osteophytes became more prominent.

Appendix 5e. Ultrasonographic assessment of cartilage thickness

	Placebo (n=35)	1st Exp (n=36)	2nd Exp (n=32)		1st/Pcb	2nd/Pcb	1st/2nd
MSUS (mm)	Mean±SD	Mean±SD	Mean±SD	*p*	*p*	*p*	*p*
R LC 0 m	1.86±0.45	1.79±0.41	1.74±0.43	0.711	0.706	0.424	0.639
R LC 3 m	1.68±0.36	1.8±0.45	1.95±0.39	0.090	0.303	0.026	0.241
R LC 6 m	1.69±0.45	1.87±0.5	1.9±0.47	0.072	0.310	0.018	0.207
R ICN 0 m	1.99±0.52	1.93±0.51	1.88±0.48	0.652	0.630	0.353	0.655
R ICN 3 m	1.96±0.4	2.18±0.48	2.17±0.35	0.363	0.333	0.146	0.731
R ICN 6 m	1.95±0.47	2.14±0.52	2.21±0.49	0.081	0.226	0.021	0.309
R MC 0 m	1.92±0.46	1.82±0.45	1.89±0.44	0.69	0.451	0.973	0.464
R MC 3 m	1.71±0.41	1.84±0.4	1.9±0.46	0.479	0.64	0.247	0.437
R MC 6 m	1.78±0.5	1.87±0.48	2±0.39	0.071	0.157	0.025	0.359
L LC 0 m	1.81±0.41	1.81±0.31	1.81±0.39	0.886	0.620	0.851	0.764
L LC 3 m	1.75±0.38	1.81±0.41	2.01±0.37	0.105	0.664	0.046	0.107
L LC 6 m	1.83±0.47	1.9±0.41	2.01±0.41	0.045	0.839	0.023	0.039
L ICN 0 m	2.06±0.5	1.96±0.41	1.87±0.47	0.267	0.412	0.122	0.384
L ICN 3 m	1.96±0.38	2.09±0.4	2.19±0.34	0.294	0.359	0.134	0.505
L ICN 6 m	2.04±0.59	2.2±0.48	2.2±0.42	0.222	0.408	0.083	0.338
L MC 0 m	1.88±0.45	1.83±0.43	1.83±0.41	0.901	0.826	0.648	0.809
L MC 3 m	1.73±0.36	1.94±0.36	2.01±0.37	0.151	0.203	0.064	0.500
L MC 6 m	1.91±0.54	1.93±0.43	2.02±0.38	0.474	0.590	0.239	0.457

MSUS: Musculoskeletal ultrasound; R LC: Right knee lateral condyle; R ICN: Right knee intercondylar notch; R MC: Right knee medial condyle; L LC: Left knee lateral condyle; L ICN: Left knee intercondylar notch; L MC: Left knee medial condyle; * p≤0.017.

Appendix 6. Adverse events occurrence during follow-up

3rd month	Placebo (n=34)	1st Exp (n=35)	2nd Exp (n=31)	*p*
Nausea	6	10	8	>0.05
Dyspepsia	8	7	6	>0.05
Diarrhea	5	6	6	>0.05
Constipation	3	2	5	>0.05
Headache	3	6	2	>0.05
Other	1	2	4	>0.05
6th month	**Placebo (n=30)**	**1st Exp (n=32)**	**2nd Exp (n=28)**	***p***
Nausea	4	8	6	>0.05
Dyspepsia	10	13	9	>0.05
Diarrhea	7	5	4	>0.05
Constipation	5	4	7	>0.05
Headache	4	6	9	>0.05
Other	5	3	4	>0.05