Impact of knee joint loading on fragmentation of serum cartilage oligomeric matrix protein

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Abstract
The aim of the study was to examine the effect of mechanical knee joint loading on the fragmentation pattern of serum cartilage oligomeric matrix protein (COMP). Ten healthy men ran with knee orthoses that were passive or active (+30.9 N·m external flexion moments) on a treadmill (30 minute; \(v = 2.2\) m/s). Lower-limb mechanics, serum COMP levels, and fragmentation patterns (baseline; 0, 0.5, 1, 2 hours postrunning) were analyzed. Running with active orthoses enhanced knee flexion moments, ankle dorsiflexion, and knee flexion angles \((P < .05)\). There was an increase in serum COMP \((+25\%\); pre: 8.9 ± 2.4 U/l; post: 10.7 ± 1.9 U/l, \(P = .001)\), COMP pentamer/tetramer \((+88\%\); 1.88 ± 0.81, \(P = .007)\), trimer \((+209\%\); 3.09 ± 2.65, \(P = .005)\), and monomer \((+78\%\); 1.78 ± 0.85, \(P = .007)\) after running with passive orthoses and in serum COMP \((+41\%\); pre: 8.5 ± 2.7 U/l; post: 11.3 ± 2.1 U/l, \(P < .001)\), COMP pentamer/tetramer \((+57\%\); 1.57 ± 0.39, \(P = .007)\), trimer \((+86\%\); 1.86 ± 0.47, \(P = .005)\), and monomer \((+19\%\); 1.19 ± 0.34, \(P = .114)\) after running with active orthoses. Increased fragmentation might indicate COMP release from cartilage while running. Interestingly, 0.5 h up to 2 hours after running with passive orthoses, trimer \((0.5\) hour: 2.73 ± 3.40, \(P = .029); 2 hours: 2.33 ± 2.88, \(P = .037)\), and monomer \((0.5\) hour: 2.23 ± 2.33, \(P = .007); 1 hour: 2.55 ± 1.96, \(P = .012); 2 hours: 2.65 ± 2.50, \(P = .009)\) increased while after running with active orthoses, pentamer/tetramer \((1\) hour: 0.79 ± 0.28, \(P = .029)\), and trimer \((1\) hour: 0.63 ± 0.14, \(P = .005); 2 hours: 0.68 ± 0.34, \(P = .047)\) decreased. It seems that COMP degradation and clearance vary depending on joint loading characteristics.

KEYWORDS
cartilage, COMP, fragmentation pattern, mechanical joint loading, western blot

1 | INTRODUCTION

Osteoarthritis (OA) is often diagnosed at a too late stage of disease when cartilage is already irreversibly degenerated. However, the symptomatic and radiographically diagnosable OA is preceded by an asymptomatic, “silent” preradiographic phase characterized by extensive changes in cartilage metabolism. Such critical metabolic events might be detected by OA biomarkers—components of the extracellular matrix (ECM) and/or their breakdown products, proteases, and cytokines that can be determined in serum, synovial fluid, or urine.
Cartilage oligomeric matrix protein (COMP), a noncollagenous perifibrillar adapter protein, is one of the most examined OA biomarkers. Serum or synovial COMP concentration has been shown to be elevated in patients with OA, OA progressors, and in conditions with changed or increased mechanical knee joint loading due to knee joint injury or obesity. Interestingly, in healthy humans without known risk factors for OA, a temporary increase in serum COMP levels after physical activity has also been detected. However, in a recent study, we could show that in healthy running men, the relationship between mechanical joint loading and serum COMP seems to be very complex. Although the subjects ran with significantly increased knee joint moments, the serum COMP levels after running were not significantly higher compared with running without additional joint load. A problem in many studies might be the use of commercial quantitative enzyme-linked immunosorbent assays (ELISA) that detect both intact pentameric COMP as well as fragments of them. Consequently, such ELISA do not provide any information on qualitative differences in serum COMP. It might be speculated that under pathological conditions there is a higher amount of fragmented COMP molecules that were released of the ECM. This assumption is based on studies that performed qualitative analyses of COMP in serum, synovial fluid, or tissue and that found more COMP fragments in patients with joint disorders compared with healthy humans: Neidhart et al observed higher amounts of small COMP fragments (50-70 kDa) in the synovial fluid of patients with rheumatoid arthritis (RA) (84%) and other forms of inflammatory arthritis (60%), compared with patients with OA (21%) or healthy controls (7%). Vilim et al and Åhrman et al were also able to identify a disease-specific COMP fragmentation pattern in synovial fluid of patients with OA, RA, and knee joint injury with the highest amounts of antigenic COMP fragments and a distinct COMP neoepitope in patients with knee joint injury followed by OA and RA. By using a self-made ELISA identifying COMP fragments, Lai et al found a significant increase of COMP fragments in serum of patients with OA and a correlation between the fragment level and OA grade. In addition, authors could show by western blot analyses that the majority of COMP is released as intact pentamer in healthy subjects, whereas in serum of patients with OA or RA besides full-length COMP also fragments were found. Compared with healthy humans, Laudon et al could detect fragments of ~23 kDa solely in males and females with a history of knee joint injury but not in uninjured subjects. Fragments of 50-100 kDa were exclusively found in females with previous knee joint injury. Western blots of extracts from healthy human knee joint cartilage detected primarily intact COMP molecules with a molecular mass of ~550 kDa. In addition, in OA and RA fragments with sizes of ~150 kDa and 67-94 kDa were found and a few samples also had smaller fragments (43-67 kDa). Interestingly, as determined by ELISA, the amount of COMP was similar in healthy and OA cartilage, but as shown by western blot analysis, the OA cartilage contained a higher percentage of fragments and less intact COMP. In summary, in healthy humans, COMP is primarily released as intact pentamer, whereas in patients with joint diseases or injury more degraded COMP and in particular smaller fragments are present. Beyond that, the above-mentioned studies clearly indicate that there are disease-specific differences in the fragmentation pattern of COMP and that exclusive quantitative examinations of COMP might hamper the interpretation of study results. Due to a lack of qualitative examinations, it only can be speculated whether the frequently observed increase in serum COMP level after physical activity is the result of cartilage destruction or just a normal turnover and short-term adaptation. Beyond that, it is still unknown if the COMP fragmentation pattern is different after physical activity with various joint loading. It can be hypothesized that a higher mechanical joint loading leads to an increase in COMP fragments in healthy humans. To our knowledge, no study to date has analyzed the effect of mechanical joint loading on the fragmentation pattern of COMP.

Therefore, in our study, we analyzed for the first time the effect of an increase in mechanical knee joint loading during a 30-minute running intervention on the serum fragmentation pattern of COMP. This qualitative analysis of serum COMP complemented quantitative examinations of the serum COMP concentration we already focused on in a recent study. For experimental variation of knee joint loading, pneumatically-driven knee orthoses were constructed that, when they were active, increased the external knee flexion moments during stance phase. To control the mechanical joint loading of the lower extremity, a 3D motion analysis was performed. We hypothesized that after both running interventions, more COMP fragments can be found in serum samples of healthy humans compared with resting serum samples gained before exercise. In addition, we hypothesized that a further increase in serum COMP fragments can be observed after running with increased knee joint loading compared with running without additional load.

2 | MATERIALS AND METHODS

2.1 | Subjects

From a previous study that was realized in two stages of measurement, we took after finishing the first trial period a sample of ten healthy men (age 27.6 ± 3.1 years, height 182.9 ± 6.5 cm, and body mass 80.0 ± 5.8 kg). Inclusion criteria were an age between 20 and 35 years, physical fitness, and a good fit of the orthoses. Exclusion criteria (determined by a questionnaire) were musculoskeletal disorders, acute injuries, malalignment, trauma, or surgery of the joints of the lower extremities. The study was approved by the local ethics committee, and written consent was obtained from all the participants.

2.2 | Experimental protocol

The protocol was carried out as described earlier. Briefly, running interventions took place on two different test days with at least five days of rest between both interventions (mean: 6.3 ± 1.1 rest days). Mechanical joint loading was minimized before the experiment and prior to each test day the subjects did not exercise for at least
24 hours. Before the first blood sample (baseline or pre) was drawn, subjects were seated for 30 minutes.\textsuperscript{11} When the first blood collection was finished, the running intervention took place. Subjects ran with knee orthoses (for details see Firner et al\textsuperscript{12} for 30 minutes on an instrumented treadmill (v = 2.2 m/s) (Treadmetrix, Park City, UT). On the first day, the orthoses were passive and thus applied no additional load at the knee joints. On the second day, the orthoses were pneumatically driven (active) and increased the external knee flexion moments by 30.9 ± 0.9 N·m during the first 80% of stance phase. The time of orthotic activity was determined individually from the mean time of stance phase captured while running with passive orthoses (18th minute). As a consequence, the interventions were not randomized. After running, the subjects were seated again for 2 hours and further blood samples were drawn immediately after (post), and then 0.5, 1, and 2 hours after the running intervention.

2.3 | Motion analysis

As described earlier,\textsuperscript{12} stance phase kinetics and kinematics were determined from 20 steps of the right leg at six time points (3rd, 8th, 13th, 18th, 23rd, and 28th minutes) during the 30-minute running intervention. Briefly, the ground reaction forces (GRF) and the point of force application were measured at 1000 Hz by four force strain-gauge sensing elements (MC3A-500, AMTI, Watertown, MA) that were incorporated in the treadmill. With the help of 20 retro-reflective markers, kinematic data were collected (250 Hz) with 11 high-speed infrared cameras (Vicon Motion Systems, Oxford, UK). During a neutral standing reference measurement, the femoral condyles were pointed and the anatomical coordinate systems were defined for each segment. The external knee flexion moment was reported in the proximal (ie, thigh) anatomical coordinate system. The determination of the anatomical coordinate system and the calculations of joint kinematics and kinetics were performed in detail, as previously described by Sanno et al\textsuperscript{19} and Willwacher et al.\textsuperscript{20,21}

In brief, all data curves were time normalized to the duration of the stance phase. A fourth-order recursive digital Butterworth filter was used to filter raw marker coordinates (cutoff frequency: 20 Hz) and GRF data (cutoff frequency: 50 Hz). Sagittal and frontal plane joint angles and external moments of the right hip, knee, and ankle were calculated with MATLAB (The MathWorks, Natick, MA) by using a five-segment (pelvis, thigh, shank, rearfoot, and forefoot), three-dimensional (3D) inverse dynamics model of the lower extremity.\textsuperscript{22,23} In the inverse dynamics analysis, we used the data for the segment mass, moment of inertia, and center of mass location from De Leva.\textsuperscript{24} The externally applied orthotic flexion moment was added to the calculated external knee joint moments at the points in time when the orthosis was active. The mean values of all six points of measurement were used to compare the motion analysis data between both interventions. For each subject, the mean values of 20 cycles, captured in sections of 10% of stance phase (0%-10%, 10-20%, etc), were calculated. Thus, in the end, we had 10 data points for each parameter.

2.4 | Serum COMP

Blood was collected by venipuncture (Vena cubitalis) with butterfly cannulas (BD Vacutainer Safety Lok 21G, BD; Franklin Lakes, NJ) in serum-gel monovettes (5.0-7.5 mL) (Sarstedt, Nümbrecht, Germany). Blood samples coagulated for 30 minutes (at RT) before the serum was isolated by centrifugation (3000 rpm for 10 minutes) and stored at −80°C until analysis. Serum COMP levels were measured by means of a quantitative enzyme-linked immunosorbent assay (COMP ELISA, AnaMar Medical AB, Lund, Sweden). We analyzed all samples in duplicate and made the analyses as specified by the manufacturer. Absorbance was measured at 450 nm (TriStar²; Berthold Technologies, Bad Wildbad, Germany). A calibration curve, calculated with a four parameter-regression by SigmaPlot 8.0 (Systat Software Inc, San Jose, CA) was used to determine serum COMP levels. According to the manufacturer’s specifications, the detection limit of COMP is less than 0.1 U/l and the inter- and intra-assay coefficients of variation are both less than 5%.

2.5 | Sodium dodecyl sulfate–polyacrylamide gel electrophoresis and western blotting

First, undiluted serum samples (6 μL) were electrophoresed on 4% to 15% sodium dodecyl sulfate (SDS) polyacrylamide gels under nonreducing conditions.\textsuperscript{25} Gels were electrophoretically transferred to a nitrocellulose membrane (Protran BA85; Whatman GmbH, Dassel, Germany) at 100 mA overnight at 4°C. Protein transfer was controlled by a reversible Ponceau-S staining. Membranes were blocked for 1 hour with Tris-buffered saline (TBS) containing 5% milk powder and incubated with a rabbit polyclonal antibody against COMP (1:2000)\textsuperscript{26} for 1.5 hour at RT. Afterward, membranes were treated with a horseradish peroxidase conjugated, swine polyclonal antibody against rabbit immunoglobulin G (1:2000; Dako, Glostrup, Denmark) for 1 hour at RT. Finally, the horseradish peroxidase activity was detected by chemiluminescent substrate (100 mM Tris, 1.25 mM luminol, 0.225 mM p-coumaric acid, 0.012% H₂O₂) and visualized by exposure on X-ray films (Super RX; Fujifilm, Tokyo, Japan). The intensity of protein bands was analyzed using Image J (National Institutes of Health, Bethesda, MD). In all 10 western blots, three bands above 250 kDa, representing intact COMP pentamer (~524 kDa),\textsuperscript{4} fragmented tetramer (~400 kDa), and trimer (~300 kDa) could be found. A further band at ~100 kDa, associated with monomeric COMP was also detectable.\textsuperscript{15} Unfortunately, in most cases, it was not possible to draw a clear separate line between the pentamer and tetramer band so that we had to analyze them together. Furthermore, in half of the cases, the bands (including each time point of measurement) of various molecular mass had different optimal exposure times (see Figure S1 for western blots with different exposure times). Consequently, we had to analyze them on different western blots. COMP pentamer/tetramer and trimer band intensities were analyzed on blots with an exposure time of 30 seconds and COMP monomer fragments on blots with longer exposure times up to 8 minutes. However, due to the fact that data were expressed as a change of intensity relative to the
baseline, this can be considered as nonproblematic. As control, human COMP (strep-tag) purified from HEK 293 EBNA-cells was used.

2.6 Statistical analysis

Statistical analyses were performed with Statistica 7.1 (StatSoft GmbH, Hamburg, Germany) and SPSS Statistics 22 (IBM Corporation, New York). Normal distribution of variables was tested with the Kolmogorov-Smirnov test. Sphericity was checked using Mauchly’s sphericity test. A two-way (intervention and time) analysis of variance with repeated measurements and Duncan’s multiple range test for post hoc analysis were performed to detect significant differences in serum COMP concentrations and mechanical joint parameters. Changes in signal intensity in western blots were identified by the Wilcoxon signed-rank test. Variables are described as mean ± standard deviation (SD). Significance was tested at a level of α = 5% for all statistical procedures.

3 | RESULTS

3.1 Serum COMP concentration

The serum COMP baseline concentration was 8.9 ± 2.4 U/l on average before running with passive orthoses and 8.5 ± 2.7 U/l prior to running with active orthoses (Figure 1). There were no significant differences between the baseline levels.

Immediately after running with passive orthoses, the serum COMP level increased by 25% (pre: 8.9 ± 2.4 U/l; post: 10.7 ± 1.9 U/l, P = .001) and after running with active orthoses by 41% (pre: 8.5 ± 2.7 U/l; post: 11.3 ± 2.1 U/l, P = .001). In turn, half an hour after running with active orthoses the serum COMP level decreased significantly (post: 11.3 ± 2.1 U/l; 0.5 h post: 9.6 ± 2.5 U/l, P = .002), whereas at the same time of measurement after running with passive orthoses, the COMP level did not change significantly. One and two hours after both running interventions, the determined COMP levels did not differ significantly from the preceding time points. In relation to the baseline level, the serum COMP concentration remained elevated 0.5 (P = .037) and 2 hours (P = .036) after running with active orthoses. Comparing both interventions, at no point of measurement there were significant differences between the serum COMP levels.

3.2 Serum COMP fragmentation pattern

COMP molecules or fragments reached its maximum signal intensity in western blots immediately after both running interventions, with the exception of COMP monomer, reaching its highest intensity 2 hours after running with passive orthoses and half an hour after running with active orthoses (Figure 2).

Interestingly, in relation to the baseline level gained before intervention (1.0), there was a significant increase in COMP pentamer/tetramer (1.88 ± 0.81; z = -2.70, P = .007), trimer (3.09 ± 2.65; z = -2.80, P = .005), and monomer (1.78 ± 0.85; z = -2.70, P = .007) after running with passive orthoses. This was more pronounced than the significant increase in COMP pentamer/tetramer (1.57 ± 0.39; z = -2.70, P = .007) and trimer (1.86 ± 0.47; z = -2.80, P = .005) and the not significant change of monomeric COMP (1.19 ± 0.34; z = -1.58, P = .144) observed after running with active orthoses (Figure 3A,B). Half an hour after running with passive orthoses, COMP pentamer/tetramer significantly decreased (post vs 0.5 hours: z = -2.70, P = .007) to a level that was no longer significantly different compared with the baseline (1.26 ± 0.83; z = -0.56, P = .575) while trimer (2.73 ± 3.40; z = -2.19, P = .029) and monomer (2.23 ± 2.33; z = -2.70, P = .007) remained significantly elevated and COMP monomer even showed a further increase compared with the previous point of measurement (Figure 3A). On the contrary, half an hour after running with active orthoses, COMP pentamer/tetramer (0.94 ± 0.37; z = -0.25, P = .803) and trimer (0.93 ± 0.25; z = -0.87, P = .384) decreased significantly (post vs 0.5 hour: z = -2.50, P = .012 and z = -2.80, P = .005) below the baseline level so that they were no longer significantly different compared with the baseline while COMP monomer remained almost unchanged (1.20 ± 0.89; z = -0.36, P = .718). Thereafter, meaning 1 hour after running with active orthoses, COMP pentamer/tetramer (0.79 ± 0.28; z = -2.19, P = .029) and trimer (0.63 ± 0.14; z = -2.80, P = .005) decreased significantly below the baseline and, even if not significant, the signal intensity of COMP monomer also dropped below the baseline (0.95 ± 0.61; z = -1.38, P = .168). The decrease of COMP trimer was also significant compared with the previous point of measurement (0.5 vs 1 hour: z = -2.29, P = .022). Two hours after running, COMP trimer (0.68 ± 0.34; z = -1.99, P = .047) remained significantly decreased while pentamer/tetramer (1.06 ± 0.44; z = -0.36, P = .719) and monomer (1.17 ± 0.91; z = -0.25, P = .803) showed a slight but not significant increase compared with the baseline (Figure 3B). The increase of COMP pentamer/tetramer was significant compared with the previous point of measurement (1 vs 2 hours: z = -2.09, P = .037). In contrast, 1 hour after running with passive orthoses, COMP monomer (2.55 ± 1.96; z = -2.50, P = .012)
continued to increase to a level significantly different from the baseline. This increase remained significant until the end of the measurement 2 hours after running (2.65 ± 2.50; z = −2.60, P = .009). In contrast, trimeric COMP (2.22 ± 2.37; z = −1.48, P = .139) was 1 hour after intervention still elevated but not in a significant manner. Two hours after running with passive orthoses, COMP trimer (2.33 ± 2.88; z = −2.09, P = .037) reached again a significantly higher level compared with the baseline. COMP pentamer/tetramer remained 1 (1.13 ± 0.61; z = −0.36, P = .719) and 2 hours (1.19 ± 0.91; z = −0.15, P = .881) after the intervention slightly, but not significantly, elevated from the baseline level (Figure 3A).

### 3.3 Joint mechanics

Comparing both interventions, in the sagittal plane, there were no significant differences in the external hip and ankle joint moments, but higher external knee flexion moments could be found while running with active orthoses during 30% to 70% of stance phase (30%–40%: P = .034; 40%–50%: P = .026; 50%–60%: P = .017; 60%–70%: P = .020) (Table 1). The maximum knee flexion moment was on average increased by 16.0% ± 3.0% through the orthotic flexion moment, acting from 10.8% ± 3.4% to 90.6% ± 9.1% of stance phase. In the frontal plane, at none of the analyzed joints did the external moments differ significantly. Regarding joint kinematics, in the sagittal plane greater ankle dorsiflexion (90%–100%: P = .039) and knee flexion angles (70%–80%: P = .036; 80%–90%: P = .023; 90%–100%: P = .017) occurred while running with active orthoses, whereas the hip joint angles did not show significant differences. In the frontal plane, at none of the analyzed joints, the angles differed significantly.

In summary, serum COMP concentrations and fragment levels, except for COMP monomer, reached its maximum immediately after both running interventions. Running with active orthoses and thus higher external flexion moments acting on knee joints led to a more...
pronounced increase in serum COMP levels, even though not significant, compared with running without additional knee joint load. On the contrary, increase in COMP fragment levels was less pronounced immediately after running with additional knee joint load and fragment levels even dropped below baseline at later time points. In contrast, within 2 hours after running without additional knee joint load, COMP trimer and monomer remained elevated and monomeric COMP even showed a further increase with time.

4 | DISCUSSION

In the present study, we examined for the first time the short-term effect of an enhanced mechanical knee joint loading during 30 minutes of running on the fragmentation pattern of COMP.

As already demonstrated in a recent study, the mean serum COMP concentration, analyzed using a commercially available ELISA, was significantly increased immediately after both running interventions. Comparing these results with western blot analysis, we could also detect—except for monomeric COMP after running with additional knee joint load—a significant increase of COMP molecules and fragments in serum after both running interventions. These results might give an indication for a mechanical release of COMP from the ECM of articular cartilage due to higher mechanical joint loading during physical activity. This would be in line with the higher OA risk of humans that are exposed to a permanently increased or changed joint load, as already shown for injury, malalignment, or overweight.

The mechanism for the increase of both total serum COMP as well as COMP fragment concentrations in healthy and diseased humans after physical activity is not yet fully understood. COMP molecules in the serum may not only originate from articular cartilage because COMP is also expressed, though in much smaller amounts, in tendon, intervertebral disc, meniscus, and ligament. Furthermore, the synovial fluid has to be considered as a relevant compartment between articular cartilage and serum and could thus contribute to the serum COMP level. Interestingly, 30 minute one-legged knee-extension exercise in subjects with OA resulted in a local decrease in the COMP concentration in the synovial fluid. Hyldahl et al detected that a decrease of COMP in synovial fluid measured in recreational runners 15 minutes after a 30-minute running intervention correlated with the increase of serum COMP observed immediately after running. This observation suggests that COMP in the synovial fluid was extruded into the extra-articular space, the blood, or the lymphatic system during mechanical loading of the joint.

The serum levels of COMP and COMP fragments were supposed to represent a marker of articular cartilage degradation. However, we do not assume that the increase in serum COMP after our running protocols with a passive or active orthosis is an indicator of cartilage destruction but rather a normal turnover or an acute short-term adaptation of the cartilage extracellular matrix. The running interventions in our study were also not strenuous activities that one would expect a significant destruction of cartilage tissue. However, it remains to be determined if an increase in serum COMP
concentration after long-term or more strenuous interventions could at least be partially due to degenerative processes.

Both running interventions resulted in a similar fragmentation pattern, meaning that the detected fragments were of the same size, but on the other hand, the kinetic was different. Half an hour up to two hours after running, differences became particularly clear with COMP molecules and fragments increasing or decreasing relative to baseline to a varying extent at different points in time. In serum COMP levels gained by ELISA, such differences could not be ascertained and at no point of measurement COMP concentrations differed significantly between both interventions.

It is interesting to note that half an hour after running without additional knee joint load, COMP trimer and monomer remained significantly elevated in blood serum while COMP pentamer/tetramer showed a significant decrease. One hour after running, COMP monomer, and 2 hours after intervention, COMP monomer and trimer were still significantly elevated. This might indicate that COMP pentamer/tetramer is further degraded to trimeric and monomeric COMP fragments in blood serum over time. However, there seem to be differences in COMP degradation and removal from blood serum between both interventions because half an hour after running with additional load, COMP pentamer/tetramer and trimer decreased significantly and they even dropped significantly below the baseline one hour after the intervention — although there was no significant change in COMP monomer.

Several enzymes, including matrix metalloproteinases (MMPs), have been identified as degrading COMP. Their release by chondrocytes is stimulated by cytokines. A significant increase in proinflammatory cytokines like interleukin-6 or tumor necrosis factor- could be observed in blood serum during and/or after long-distance running. Interestingly, loading characteristics seem to have an influence on this inflammation-associated biochemical response, and there seems to be a threshold above which loading becomes detrimental. Marathon runners that the faster the run, the higher the increase in MMP-3 serum levels. In multistage ultramarathon runners, Mündermann et al found a positive correlation between changes in serum COMP and MMP-3 levels. Beyond that, patients with a rupture of the anterior cruciate ligament, showing changes in joint kinematics and knee joint kinematics, revealed a pronounced inflammatory response.

In our study, we also could detect significant changes in knee joint mechanics while running with active orthoses with higher external knee flexion moments and greater knee flexion angles. Consequently, it might be speculated that running with active orthoses led to a more pronounced inflammatory response than running without additional knee joint load. Thus, higher levels of catabolic enzymes in serum might explain a faster degradation and removal of COMP from blood serum during and after running with additional knee joint load. This could also explain the significant decrease of serum COMP concentration that took place half an hour after running with increased knee joint loading. Interestingly, Mündermann et al also showed in marathon runners with faster finishing times a faster return of serum COMP to prerace levels. Another explanation for a more rapid decrease of serum COMP during and after running with active knee orthoses might be the fact that the absorption of fluid across the synovial lining increases as a function of the intra-articular pressure (IAP). The IAP cyclically rises during exercise and net drainage out of cavity occurs at supraplasmatic pressures in flexed joints. Consequently, the observed greater knee flexion while running with active orthoses might have enhanced the positive effect of joint motion on convective transport, and thus the drainage of fluid and solutes from joint cavity into lymphatics and circulation. However, due to the fact that we did not determine COMP in synovial fluid before and after intervention, this remains a hypothesis. Furthermore, we did not collect blood samples during running exercise and can therefore not exclude that while running with additional knee joint load COMP fragments reached its maximum already at an earlier time point that we did not capture.

We quantified joint load at the knee during slow running by means of net external flexion and adduction moments. While the knee adduction moment and the combination of adduction moment and knee flexion moment have been correlated with the medial knee contact force, they might not be the ideal candidates for quantifying cartilage loading at the knee. This is because joint moments are only partly generated by bone-to-bone contact forces. Next to these contact forces, they are also generated by muscle-tendon-unit and passive tissue forces, for example, ligament forces. Furthermore, the calculation of net joint moments does not consider cocontractions of agonist and antagonist muscle groups crossing the knee. Future studies should explore the potential of the combination of individualized musculoskeletal modeling and electromyographic techniques to figure out whether a more direct estimation of cartilage loading can predict the COMP's response to locomotion interventions more precisely.

The present study has some limitations. We neither analyzed COMP degrading enzymes, nor cytokines that stimulate their secretion by chondrocytes. Thus, we only can speculate that changes in knee joint mechanics led to a more pronounced inflammatory response and higher levels of catabolic enzymes. Beyond that, it would have been interesting to analyze COMP not only in serum but also in synovial fluid and to measure serum COMP several times during the running intervention.

In summary, the present study is the first that analyzed the effect of an increase in mechanical knee joint loading while running on the fragmentation pattern of serum COMP. While running with active orthoses, significantly higher external knee flexion moments as well as greater ankle dorsiflexion and knee flexion angles occurred. Serum COMP levels showed a significant increase immediately after both running interventions but at no point of measurement they differed significantly between trials. Although there were no differences in fragmentation patterns, distinct differences in COMP kinetics could be observed.

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CONFLICT OF INTERESTS
The authors declare that there are no conflict of interests.

AUTHOR CONTRIBUTIONS
The study was designed by AN, G-PB, and SF. MdM, AN, FZ, and SF collected and processed the data. SW performed the MATLAB analysis. JH and SF conducted the SDS-PAGE and western blotting. SF performed the statistical analysis and drafted the manuscript. AN, G-PB, JH, and FZ contributed to the manuscript. All authors read and approved the final manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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