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Acute loading has minor influence on human articular cartilage gene expression and glycosaminoglycan composition in late-stage knee osteoarthritis: a randomised controlled trial

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Objective: Osteoarthritis (OA) remains clinically challenging. Regular physical exercise improves symptoms though it is unclear whether exercise influences cartilage at the molecular level. Thus, we aimed to determine the effect of acute loading on gene expression and glycosaminoglycan (GAG) content in human OA cartilage.

Design: Patients with primary knee OA participated in this single-blind randomised controlled trial initiated 3.5 h prior to scheduled joint replacement surgery with or without loading by performing one bout of resistance exercise (one-legged leg press). Cartilage from the medial tibia condyle was sampled centrally, under the meniscus, and from peripheral osteophytes. Samples were analysed for gene expression by real-time reverse transcriptase polymerase chain reaction, and hyaluronidase-extracted matrix was analysed for GAG composition by immuno- and dimethyl-methylene blue assays.

Results: Of 32 patients randomised, 31 completed the intervention: mean age 69 ± 7.5 years (SD), 58% female, BMI 29.4 ± 4.4 kg/m². Exercise increased chondroitin sulphate extractability [95% CI: 1.01 to 2.46; P = 0.0486] but cartilage relevant gene expression was unchanged. Regionally, the submeniscal area showed higher MMP-3, MMP-13, IGF-1Ea, and CTGF, together with lower lubricin and COMP expression compared to the central condylar region. Further, osteophyte expression of MMP-1, MMP-13, IGF-1Ea, and TGF-β3 was higher than articular cartilage and lower for aggrecan, COMP, and FGF-2. Hyaluronidase-extracted matrix from central condylar cartilage contained more GAGs but less chondroitin sulphate compared to submeniscal cartilage.

Conclusion: Acute exercise had minor influence on cartilage GAG dynamics, indicating that osteoarthritic cartilage is not significantly affected by acute exercise. However, the regional differences suggest a chronic mechanical influence on human cartilage.

ClinicalTrials.gov registration number: NCT03410745.

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systemic sampling likely does not reflect the cartilage in a single joint. Human cartilage samples can be obtained with joint replacement surgery. This single sampling point enables a prior stimulus but does not allow for longitudinal observation.

Studies subjecting human chondrocytes or cartilage explants to mechanical load have shown mechanically regulated genes within groups of matrix proteins, cytokines, growth factors, and degrading enzymes. However, conflicting results have been found likely from variations in tissue preparation and loading protocol which further complicates the extrapolation to in vivo conditions to the clinic. Thus, despite the comprehensive knowledge, the effect of exercise on human cartilage itself is unknown. Therefore, this study aimed to examine the effect of a single bout of resistance exercise on gene expression and glycosaminoglycan (GAG) content and dynamics in human OA knee cartilage. As the symptomatic benefit of exercise is well described, we hypothesise that exercise will provide a beneficial, anti-catabolic and/or anti-inflammatory response at the cellular level.

Methods

Trial design

This was a single day, two-arm, randomised clinical trial of resistance exercise compared to no exercise in patients with OA of the knee scheduled for joint replacement surgery. Primary outcomes were changes in gene expression and GAG content. No study was available for precise sample size calculation; however, a human exercise study on OA patients with direct sampling from the joint found that 29 patients with knee OA divided in two groups were sufficient for detecting a difference in intra-articular IL-10 concentrations. Accounting for possible dropouts at 10% we estimated the total need of 32 participants. The study was performed at Bispebjerg Hospital, Copenhagen, Denmark. Participants gave written informed content, and the study was approved by the Ethical Committee of the Capital Region of Denmark (H-17015563) in accordance with the Helsinki Declaration of 1975/83. The trial was registered on ClinicalTrials.gov (NCT03410745) – initially as a single trial on collagen synthesis containing 4 weeks of isotope labelling with deuterated water combined with 4 weeks of exercise intervention. However, due to recruitment issues, the trial was split into two studies, where the 4-week prospective deuterium labelling study on collagen synthesis is not a trial. We have deliberately not changed the collagen synthesis registration on ClinicalTrials.gov partly for transparency on the initial single study design, and partly to highlight that recruiting patients for long-term exercise intervention is impossible in our local setting.

Participants

Patients were recruited from the outpatient clinic at Bispebjerg Hospital orthopaedic department. Patients with OA of the knee as classified by the American College of Rheumatology (2006) were included for a knee replacement surgery by an orthopaedic surgeon who considered for participation. Patients were eligible if they were ≥40–90 years old, had primary OA, were able to perform physical exercise, had a body mass index (BMI) within 18.5–40 kg/m², and were non-smokers.

Exclusion criteria included other diseases of the joint (rheumatoid arthritis, gout, psoriatic arthritis), inflammatory diseases, diabetes, cancer, previous knee surgery, recent trauma to the knee, participation in regular strenuous exercise, or required an interpreter. Furthermore, use of non-steroid anti-inflammatory drugs (NSAIDs), oral corticoid-steroids or intraarticular injection within 3 months, disease modifying anti-rheumatic drugs (DMARDs), chemotherapy, or other immuno-suppressive medicine deemed the patient ineligible.

Intervention

Exercise was supervised by a physician and began with a light warm-up on an ergometer bike for 5–10 min. In a leg-press machine (Techno Gym, Cesena, Italy), participants were seated upright with the hip hinged 90°. To ensure proper load on the knee of interest, one-legged exercise was performed with the working leg extending from 100° to near fully stretched against a vertical plate, and the resting leg positioned on a horizontal plate. Due to safety concerns of the overnight fasted participants with knee pain, we performed a 5 repetition maximum (RM) test as an indirect measure of maximum strength to calculate the corresponding 1 RM. Three sets of eight repetitions at 70% 1 RM and three sets of four repetitions at 80% 1 RM were performed at a controlled pace.

Randomisation

Participants were randomly allocated 1:1 to one of the two groups using the mininisation software MinimPy version 0.3 (Python Software Foundation, Beaverton, OR, USA), stratified by sex, age (<70 and 70+ years), and BMI (18.5–25, 25.1–30, and 30.1–40 kg/m²). Due to the nature of the intervention, the participants were unblinded, however, to the best of our knowledge the outcome measurements cannot be influenced by own will. Surgeons were unaware of the allocated group during surgery, and messenger ribonucleic acid (mRNA) and GAG data were obtained blinded.

Tissue collection

During knee replacement surgery, the leftover tibia plateau was collected and immediately transferred on ice to the laboratory. From the medial tibia condyle, several full-thickness cartilage biopsies (30–150 mg) were sampled centrally and under the meniscus. By cutting with a scalpel, the subchondral bone was removed from biopsies. From osteophytes, slices (10–120 mg) were sampled using a scalpel. The cartilage was snap-frozen within 15 min from surgery in liquid nitrogen and kept on –80°C until further analysis.

Gene expression analysis

From the cartilage biopsies and slices, mRNA expression was measured as previously described. In brief, total RNA was isolated from cartilage and osteophyte tissue by homogenisation in a guanidine thiocyanate/phenol solution, before being precipitated, and further extracted using the RNeasy MinElute Cleanup Kit (Qiagen, Hilden, Germany) as per manufacture’s protocol. The total RNA concentration was determined by the Ribogreen assay (Molecular Probes Inc., Eugene, OR, USA). 50 ng total RNA was reversed transcribed to complementary deoxyribonucleic acid (cDNA), and target mRNAs were quantified using real-time reverse transcriptase polymerase chain reaction (RT-PCR). Primers are given in Supplementary Table S1. Ct values were converted to cDNA molecules using a standard curve of DNA oligos (Ultramer, Integrated DNA Technologies Inc., Leuven, Belgium) with a DNA sequence corresponding to the expected PCR product. No sample specific inhibition was seen comparing efficiency of the individual sample PCR reactions with the standard curve using the three-parameter logistic model. Expressed values were normalised to internal control of large ribosomal protein P0 (RPLP0), which was then validated (no difference between groups and low variation) using

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glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The RPLP0-normalized values are expressed as ratio to the total geometric mean for all samples of the central region.

**Extraction and quantification of glycosaminoglycans**

The central and sub-meniscal cartilage biopsies (all osteophyte tissue was used for RNA) were sliced, weighed, freeze-dried, and weighed again (dry weight). Next, GAGs were extracted by overnight treatment with hyaluronidase (H3506, Sigma, Darmstadt, Germany) [5 U/ml in 0.05 M sodium acetate and 0.15 M NaCl (pH = 6)] at 37°C. The samples were centrifuged, and the supernatant collected, aliquoted, and stored at −80°C until further analyses.

GAG content was measured using the dimethyl-methylene blue (DMMB) assay. The hyaluronidase-treated cartilage biopsies were washed with isotonic NaCl, and then treated overnight at 60°C with 0.125 mg/ml papain (P3125, Sigma, Darmstadt, Germany) in PBE chemo-luminescence. On positively charged nylon membranes a standard curve of chondroitin sulphate (CS) C (C4384, Sigma, 540 nm wavelengths (subtracted) and compared to a known weight of the cartilage sample. All samples are expressed relative to background with ImageJ software and normalised to initial dry weight. 

**Results**

The trial was completed as the 32 participants were recruited between May 2018 and September 2019 (Fig. 1). There are similar characteristics in the two groups (Table I). No harms were reported during the exercise intervention.

**Gene expression and loading intervention**

In the intervention group, the loaded weight reported as the 5RM test showed a median of 110 kg [95% CI: 90–120 kg] (Fig. S1). For both groups, low amounts of RNA were extracted for articular cartilage with means ± SD of 3.1 ± 1.6, 2.6 ± 1.5, 2.9 ± 1.6, and 3.2 ± 2.0 ng/mg tissue, while more RNA was extracted for osteophytes 16.7 ± 6.8 and 17.3 ± 28.8 ng/mg tissue (Fig. S2). Four samples were of insufficient quality (two in each group), and in total, 23 genes were assessed of which 13 were detected sufficiently for analyses (Table S2). We found no difference in GAPDH, thus validating the use of RPLP0 for normalisation [Fig. 2(A)]. On gene expression, the exercise intervention marked with striped fill pattern on bars in Fig. 2 did not show a significant effect on any target. As it was unexpected that no targets were affected by acute loading, the immediate early genes fos-proto-oncogene (FOS) and early growth response 1 (EGR1) were subsequently analysed. They both showed a minor but statistical difference after the loading intervention for FOS [95% CI: 0.21 to 0.99; P = 0.048] and for EGR1 [0.24 to 0.95; P = 0.034], supporting that late-stage OA cartilage is able to respond to the acute loading intervention.

**Regional gene expression**

In Fig. 2, the submeniscal cartilage region (light grey coloured bars) and osteophytes (dark grey coloured bars) are compared to the central condyle region (white coloured bars). Compared to the central region we found significantly higher fold expression of matrix metalloproteinase 3 (MMP-3) at 2.9 fold [1.7 to 5.0; P < 0.0001], MMP-13 at 3.9 fold [1.4 to 10.7; P = 0.0054], insulin-like growth factor 1 (IGF-1Ea) at 2.6 fold [1.2 to 5.6; P = 0.0127], and connective tissue growth factor (CTGF) at 1.8 fold [1.3 to 2.4; P = 0.0002], combined with lower fold expression of lubricin (PRG4) at 0.4 fold [0.2 to 0.7; P = 0.0004] and cartilage oligomeric protein (COMP) at 0.6 fold [0.4 to 0.9; P = 0.004] in the submeniscal region (Fig. 2).

Compared to the central region of articular cartilage, osteophytes showed higher fold expression of MMP-1 at 4.8 fold [2.6 to 9.2; P < 0.0001], 78.1 fold MMP-13 [25.8 to 236.1; P < 0.0001], 35.1 fold IGF-1Ea [15.0 to 82.3; P < 0.0001], 2.2 fold TGF−β2 [1.2 to 4.1; P = 0.0067] and 2.8 fold TGF−β3 [1.7 to 4.6; P < 0.0001] together with lower fold expression of aggrecan (ACAN) at 3.2 fold [2.2 to 4.6; P < 0.0001], 0.23 fold COMP [0.15 to 0.35; P < 0.0001], and 0.5 fold fibroblast growth factor 2 (FGF-2) [0.4 to 0.7; P < 0.0001] (Fig. 2).

**Glycosaminoglycans**

From the central region, hyaluronidase treatment extracted a mean of 17.5 μg/mg dry weight (d.w.) GAGs compared to 10.9 μg/mg d.w. from submeniscal regional [1.5 to 11.7; P = 0.012] with no effect of exercise intervention [Fig. 3(A)]. Levels of GAGs remaining in the cartilage biopsies were not significantly different with...
CONSORT diagram. The flow of participants through the study.

**Parameter Control (n = 15) Exercise (n = 16)**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control (n = 15)</th>
<th>Exercise (n = 16)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years ± SD)</td>
<td>67.8 ± 8.0</td>
<td>69.5 ± 7.2</td>
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<tr>
<td>Body mass index (kg/m² ± SD)</td>
<td>29.7 ± 3.2</td>
<td>29.2 ± 5.4</td>
</tr>
<tr>
<td>Sex (percentage female)</td>
<td>67%</td>
<td>50%</td>
</tr>
<tr>
<td>Kellgren–Lawrence grade (score [range])</td>
<td>3.6 [3–4]</td>
<td>3.4 [3–4]</td>
</tr>
<tr>
<td>5 repetition maximum (kg median with 95% CI)</td>
<td>N/A</td>
<td>110 [90–120]</td>
</tr>
<tr>
<td>Time from exercise to tissue freezing (h:mm ± SD)</td>
<td>N/A</td>
<td>3:24 ± 0:37</td>
</tr>
</tbody>
</table>

N/A: Not applicable. Values are means if not stated otherwise.

**Table I**

Participants’ characteristics
95.9 µg/mg d.w. centrally and 107.5 µg/mg d.w. submeniscally and with no effect of exercise intervention \([\text{Fig. 3(B)}]\). Total GAGs were not significantly different with 113.4 µg/mg d.w. centrally and 118.4 µg/mg d.w. submeniscally with no effect of exercise intervention \([\text{Fig. 3(C)}]\). In the supernatant after hyaluronidase treatment, CS content showed higher extractability after loading \([1.01 \text{ to } 2.46; P = 0.0486]\) with higher amount extracted to the supernatant from submeniscal region compared to central region \([1.24 \text{ to } 2.41; P = 0.0023]\) \([\text{Fig. 4(A)}]\). KS showed significant interaction \([0.81 \text{ to } 0.99; P = 0.045]\) \([\text{Fig. 4(B)}]\).

Discussion

**Acute loading has minor influence on chondrocyte gene expression**

This randomised controlled trial examined the effect of acute exercise in vivo on gene expression from human OA chondrocytes from three regions of the tibia plateau. As we found no cartilage genes responsive to the intervention, we examined two immediate early genes, which both did respond to the loading intervention. This is somewhat in contrast to upregulation of expression in multiple genes that has been reported from a human in vitro study\(^{16}\). On the other hand, this is the first study to examine the effect of exercise on gene expression directly in joint cartilage in humans in vivo, and thus, we are unable to compare our results directly. Our data suggest that in humans with OA, chondrocyte gene expression of cartilage relevant genes is in fact not regulated in a detectable manner by this single bout of exercise setup within the given timeframe.

In humans subjected to acute exercise, biomarker studies on serum\(^1\) and SF\(^18\) exist, and although COMP has been widely examined for breakdown of cartilage, COMP is also expressed in synovial cells\(^19\), complicating the measurements on SF. We did not detect any effect of loading on chondrocyte gene expression. Decreased levels of SF COMP has been reported after acute exercise\(^10\), although baseline measurements were made 3 months earlier, and an effect of time cannot be ruled out. Further, with acute exercise, we did not find any effect upon aggrecan expression. Similarly, in OA chondrocytes, loading did not change aggrecan expression, whereas an increase was observed in healthy...
chondrocytes. Using delayed gadolinium-enhanced magnetic resonance imaging of cartilage (dGEMRIC) as a surrogate marker for GAG content, long-term exercise provided no change in tibiofemoral cartilage with mild OA. Thus, in OA, an exercise-induced lubricin secretion from chondrocytes are uncertain at best. Further, a study applying in vivo stretching of synovial fibroblasts, found increased lubricin gene expression in healthy but not OA donors.

In our present study, similar to others, we did not detect any mRNA from interleukins (ILs). This raises the question of whether cells from other regions (i.e., synovial lining or subchondral bone) could in fact be the population responsible for IL production. Another possibility is differing tissue preparation, as one study found a marked increase in IL expression in vitro compared to in vivo. In patients with moderate OA (Kellgren–Lawrence 2–3), increased SF and perisynovial IL-10 was found in the recovery after acute exercise, however, IL-10 levels were not measured before exercise. In our setup with direct visualisation of the joint capsule during surgery, too few SF samples were collectable to perform feasible SF analysis and further conclusions on IL-10 with exercise remains speculative.

In healthy human chondrocytes that are exposed to load, MMP-1 is either unchanged or decreases together with MMP-3 and MMP-13 due to increasing IL-21,33. However, in OA there was no change in MMP-1 or MMP-3, similar to our data. Further, in the present study we barely detected MMP-1, consistent with one study but in contrast to others. Thus, our human exercise data are in line with the in vitro studies, and MMPs are not influenced within our setup. Finally, ADAMTS-4 was undetected, and ADAMTS-5 was found in too low amount contrary to others. From our data, additional conclusions on ADAMTS are speculative. Currently, exercise did not lead to increased expression of breakdown enzymes nor cytokines, and consequently, in patients with late-stage OA, the performed exercise intervention appears safe for cartilage.

One limitation of the study is that only 23% of the patients screened were eligible for participation, thus limiting the generalisability of the study. However, the comprehensive eligibility criteria optimised the possibility of identifying an effect of exercise, and the trial participants are believed to represent the most likely to respond to exercise. Hence, an effect in post-traumatic OA cannot be excluded. Another limitation is the intervention itself. The effect of exercise may emerge later – although the genes of interest are reported to change after the 3-h timeframe. Our intervention could be inadequate as a stimulus as the participants did not perform an all-out resistance exercise bout. However, the performed exercise uses moderate to hard load, and thus, the cartilage should be loaded sufficiently, which is supported by the loading effect in FOS and EGR1. Further, despite our participants being overnight fasted and untrained, all completed the protocol without any harm, as has been reported previously, thus making the intervention clinically possible and applicable. Finally, a single bout of resistance exercise might not be enough, as months of exercise intervention clinically possible and applicable. Finally, a single bout of resistance exercise might not be enough, as months of exercise tended to show a beneficial response on cartilage biomarkers. Potentially, the slight and insignificant difference in sex distribution between the two groups would have influenced the results as women in general are found to have more severe OA. In counter to this argument, the analysis of KL-score between males and females in the present study were similar. Further, in the present study we did determine the outcome result before and after exercise, but due to the inability to sample more than one cartilage sample within a single individual, we cannot specifically know what the true baseline values in cartilage were. Given this drawback in human
studies we still find it important to measure cartilage characteristic as done in the present study.

**Regional differences in chondrocyte gene expression**

As the submeniscal area on the medial plateau experience higher load than central, we sampled from both regions. Our data showed increased MMP-3, MMP-13, IGF-1Ea, and CTGF, and decreased lubricin and COMP in the submeniscal area. Work from our lab confirms similar regional differences for MMP-3 and lubricin. Thus, it appears that breakdown, fibrosis, and reduced anabolism dominate submeniscally. In the current study, cartilage fibration was universally found centrally, whereas submeniscal cartilage conditions varied from appearing healthy to a complete loss. This appearance is also reported by others, who showed increased MMP-3, MMP-13, and IGF-1Ea in undamaged cartilage (corresponding to submeniscal) from the medial tibia plateau, and decreased COMP. From femur condyles, results varies: some studies found differences in MMP-3, and MMP-13, while others did not. We found no difference in expression of aggrecan or COL2A1, similar to many, although decreased aggrecan has been found centrally.

Osteophytes are bony outgrowths covered by fibro-cartilage, and compared to articular cartilage, we found decreased aggrecan, COMP, and FGF-2 and increased MMP-1, MMP-13, TGF-β2, TGF-β3, and IGF-1Ea compatible with osteophyte growth and ossification capability. Very limited work has been done in humans on chondrocyte gene expression from osteophytes, but similar increases in TGF-β and unchanged FGF-2 have been found, while IGF-1 was found expressed in osteophytes, the level was not compared to articular cartilage. Thus, osteophyte gene expression differs from articular cartilage, and the pattern conforms with osteophyte growth, remodelling, and ossification capability supporting the underlying bony growth. Hence, care should be made during cartilage tissue sampling of an entire joint surface for RNA analysis.

**Glycosaminoglycans are slightly affected by acute loading and regionally different**

The total content of GAGs in cartilage and SF is unlikely to change sufficiently for robust detection, however, we examined the potential influence of acute exercise upon GAG dynamics. We used hyaluronidase in rather low amounts to assess the extractable amounts of GAGs as a marker for looseness in the matrix. We examined the GAG composition of the hyaluronidase supernatant further and found minor effects of exercise with increased extractability of CS and an interaction of KS. Thus, acute loading could potentially affect some GAG dynamics, and we cannot exclude that more pronounced dynamic changes would have been observed if we had sampled sometime later than the 3.5 h.

Regionally, more GAGs were extracted from the central region, which showed macroscopic fibrillation universally, and thus, likely having more loosely bound matrix. Others too, have shown less GAGs left in the damaged medial femur compared to the lateral. Aggrecan is linked to HA and contains a smaller KS rich section followed by two large CS rich sections, and as aggrecan gene expression is regionally similar, breakdown could be the dominant factor. Finally, more CS were extracted submeniscally with equal KS extractability. Combined with the results on regional gene expression, a careful suggestion could be that matrix breakdown by MMPs, ossification due to IGF-1 and CTGF signalling, and reduced cartilage matrix formation of COMP and lubricin dominates under the meniscus in areas where cartilage still exists. Enzymatic degradation of aggrecan would release CS rich sections sequentially while keeping most KS attached, and in support, others have found less CS left in areas with more damage. As submeniscal cartilage varied from healthy looking to complete subchondral induration, this process seems the likely future outcome for all submeniscal cartilage had surgery not been performed. Our data suggest regional differences and a possible acute effect of in vivo loading despite the absolute amounts of GAGs involved being rather small, and thus, further studies are necessary to uncover exercise-induced GAG dynamics.

Taken together, we found minor effects on GAG extractability. Thus, the beneficial response found in healthy cartilage appears not to be reflected in late-stage human OA within the given timeframe. This suggests clinical improvements of exercise are likely a result of the known extra-articular musculo-skeletal adaptations. Nevertheless, from the lack of a major effect, exercise appears safe in late-stage OA, albeit not beneficial per se. Finally, regional differences in chondrocyte gene expression indicate an unfavourable environment under the meniscus, possibly leading to subchondral

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**Fig. 4**

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Immuno-assay. GAG measurements in the supernatant after hyaluronidase treatment. All samples are expressed relative to the geometric mean of the central region. A: Keratan sulphate B: Chondroitin sulphate. Each data point is means of duplicate analyses and error bars represent geometric means with 95% CI (back transformed) shown on logarithmic scale.
exposure frequently observed is this region. In conclusion, acute exercise did not influence expression of cartilage relevant proteins and had minor effects on GAG dynamics, indicative of a relatively stable pathological cartilage in OA. The major regional cartilage differences demonstrated in this study indicates a chronic mechanical influence upon joint cartilage in humans.

Contributions

AJ, PS & MK conceptualized and designed the study. AJ acquired data and drafted the article. PS & AJ performed the analysis and interpretation of RNA data and DMMB assay. BDV performed the analysis of immune assay. All authors reviewed the manuscript critically for important intellectual content and approved the final version to be submitted. AJ and MK take responsibility for the integrity of the work as a whole, from inception to finished article.

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Declaration of competing interest

BDV and JR are owners of GLX Analytix, a company that studies glyocalyx shedding in relation to disease. GLX Analytix has had no influence on the design, data handling and decision to publish. The remaining authors report no conflicts of interest.

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Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.joca.2023.01.317.

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