Ultrasonic evaluation of acute impact injury of articular cartilage in vitro

T. Virén †‡*, M. Timonen †, H. Tyrväinen †, V. Titu §, J.S. Jurvelin †, J. Töyräs † ||

† Department of Applied Physics, University of Eastern Finland, Kuopio, Finland
‡ Department of Orthopaedics, Traumatology and Hand surgery, Kuopio University Hospital, Kuopio, Finland
§ Institute of Biomedicine, Anatomy, University of Eastern Finland, Kuopio, Finland
|| Department of Clinical Neurophysiology, Kuopio University Hospital, Kuopio, Finland

SUMMARY

Objective: The aim of the study was to investigate whether high frequency ultrasound technique, originally designed for arthroscopic use can be utilized to detect traumatic cartilage injuries.

Methods: A total of four intact osteochondral plugs were prepared from eight patellas for parallel comparison (total of 32 plugs). The plugs were injured by dropping an impactor on them from heights of 2.5 cm, 5.0 cm, 10.0 cm and 15.0 cm (corresponding to impact energies of 0.12, 0.25 0.50 and 0.74 J, respectively), in a custom made dropping tower. The samples were imaged with a high frequency (40 MHz) ultrasound device before and after the injury. Reflection coefficient (R), integrated reflection coefficient (IRC), apparent integrated backscattering (AIB) and ultrasound roughness index (URI) were determined for each sample.

Results: Injuries invisible to the naked eye could be sensitively detected via the decreased values of the ultrasound reflection parameters (P < 0.05). Furthermore, a decreasing trend was detected in the values of R and IRC as the momentum of the impactor increased. The values of AIB were significantly lower for samples injured by dropping the impactor on the cartilage from heights of 2.5 cm and 15 cm but the URI values were similar in intact and injured cartilage. Histological analysis of the cartilage samples revealed that the injured cartilage exhibited depletion of the cartilage surface proteoglycans but the structure of collagen network was almost normal.

Conclusions: Quantitative ultrasound imaging enables the detection of minor visually non-detectable cartilage injuries. As the present technique is feasible for arthroscopic use it might have clinical value in the evaluation of cartilage lesions during arthroscopy e.g., after tear of the anterior cruciate ligament.

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Introduction

Osteoarthritis (OA) is a degenerative joint disease characterized by progressive degeneration of the articular surfaces and remodeling of subchondral bone, leading to pain and loss of joint function. Although the pathogenesis of OA is still unclear, several factors that affect the development of OA have been identified. Impact injuries of articular cartilage generated by joint traumas are a known cause of degeneration of articular cartilage and the appearance of posttraumatic OA. Thus, for effective treatment as well as prevention of the degeneration of articular surfaces, it is important to make an early diagnosis of cartilage damage. However, detection of non-macroscopic cartilage surface damage is challenging with current clinical imaging techniques. Articular cartilage is not visible in X-ray images and the high costs and limited resolution of magnetic resonances imaging (MRI) limit their application in the microscopic assessment of cartilage. Currently, arthroscopic examination of the joint is a routine procedure for evaluation of the severity of cartilage injury. However, arthroscopy enables only a visual evaluation of the cartilage surface although it can be combined with subjective palpation of the cartilage stiffness. A recent study reported that the majority of experienced arthroscopists considered that the differentiation between low-grade and high-grade cartilage lesions is challenging and in need of improvement. In the same study, 75% of the arthroscopists stated that a more objective measurement would be very useful or somewhat useful. Furthermore, inter-observer reliability of arthroscopic grading has been reported to be poor. Thus, more sensitive imaging methods are needed to detect acute cartilage lesions.

Ultrasound is a widely used medical imaging modality. It is based on measurements of reflection and scattering of ultrasound that occurs at the interfaces between tissues with different acoustic
impedances; these are determined by tissue density and mechanical properties. By measuring the pressure of an ultrasound pulse reflected at the interface, the difference in the acoustic properties of the materials can be approximated.

Numerous ultrasound techniques have been described in the literature for quantification of the integrity of articular cartilage. The techniques are based on the measurements of ultrasound velocity, attenuation and scattering inside cartilage reflection and scattering from the cartilage surface and measurements of cartilage thickness. With ultrasound mechanical or enzymatic degradation as well as spontaneous degeneration of the cartilage surface can be detected. Furthermore, ultrasound backscattering from the inner structures of cartilage has been reported to be sensitive to changes in the collagen network in cartilage. By measuring the pressure of an ultrasound pulse at the interface, the difference in the acoustic properties of the materials can be approximated.

We have recently introduced an arthroscopic ultrasound technique for evaluation of the integrity of articular cartilage. The technique is based on the use of a clinical high frequency intravascular ultrasound (IVUS) device and catheters (Boston Scientific, San Jose, CA, USA). This novel arthroscopic ultrasound technique can assess in a sensitive manner cartilage thickness, artificial degeneration of the cartilage surface as well as abnormal structures in the collagen network inside surgically repaired cartilage. Furthermore, with the arthroscopic ultrasound technique, the severity of mechanical degradation and depth of the mechanically induced cartilage lesion in a bovine knee joint could be sensitively evaluated during arthroscopy. Importantly, quantitative ultrasound imaging has already been applied during arthroscopy of human knee.

The aim of this study was to investigate the ability of the arthroscopic ultrasound technique to detect acute injury of articular cartilage after mechanical impact. We hypothesized that mechanical impact would damage the cartilage collagen network of superficial cartilage and that this could be detected in the ultrasound measurement as a decrease in cartilage surface reflection, an increase of cartilage surface roughness and an increase of scattering inside the cartilage.

Materials and methods

Intact bovine (18-months-old) knee joints (number of joints = 8) were obtained from a local slaughterhouse (Atria Oyj, Kuopio, Finland) and stored at +4°C until the experiment. The joints originated from eight different animals and were treated as independent specimens in the statistical analysis. The knees were opened within two days post mortem and osteochondral plugs (diam. = 25.4 mm) were prepared from lateral proximal patellae. Subsequently, the discs were divided into four pieces and a smaller osteochondral plug (diam. = 6.1 mm) was punched from each piece. Since the samples were prepared from an area with a diameter of 25.4 mm, the tissue characteristics of samples prepared from each patella were assumed to be similar. One sample from each patella was included in each sample group. The tissue adjacent to the smaller plug was used as intact control tissue in the histological analysis. All samples were immersed in phosphate buffered saline (PBS), containing inhibitors of proteolytic enzymes (5 mM disodium Ethylenediaminetetraacetic acid (EDTA) and 5 mM benzamidine HCl), during the measurements. Disodium EDTA inhibits activity of enzymes dissolving collagen and benzamidine HCl specifically retains the proteoglycans.

Impact injury was created by dropping an impactor (weight 500 g) onto the osteochondral plug from different heights 2.5 cm, 5.0 cm, or 15 cm (corresponding to the energies of 0.12 J, 0.25 J, 0.50 J and 0.74 J at the instant of the impact, respectively) in a custom made drop tower (Fig. 1). The impactor was lifted from the sample within 1 s after the impact to prevent any major creep deformation. After the measurements samples were prepared for histological analysis. Samples were first chemically fixed in 4% (weight/volume, w/v) formaldehyde buffered to pH 7.0 for 48 h and then decalcified in 10% (w/v) EDTA in 4% (w/v) formaldehyde, buffered to pH 7.4 for 2-weeks. Subsequently, the samples were dehydrated in alcohol, filtered and embedded in Tissue-Tek III embedding wax (polymer added) (Sakura Fintek Europe, Zoeterwoude, Netherlands).

![Intact tissue](Image)

![Mechanical injury](Image)

![Injury](Image)

**Fig. 1.** Intact osteochondral samples were imaged with ultrasound and a light microscope. Subsequently, an impact injury was created on cartilage samples with a custom made dropping tower. The weight of 500 g was dropped on the cartilage samples from heights of 2.5 cm, 5.0 cm, 10 cm and 15 cm. In order to prevent any major creep deformation of the cartilage the weight was lifted from the sample within 1 s. After the injury, samples were imaged again with ultrasound and a light microscope.
Drop towers have been used to produce loads typical to the impact injuries of articular cartilage. In the study of Jeffrey et al. the impact energies of 0.049—1.69 J evoked cartilage injuries ranging from mild to severe\(^1\) (chondral and osteochondral discs, \(\text{diam.} = 5 \text{ mm}\)). Furthermore, in a recent study applying a similar drop tower as that used in the present study with an impact energy of 0.74 J created cracks which penetrate from surface to transitional zone (osteochondral discs \(\text{diam.} = 6.1 \text{ mm}\))\(^22\). Surface cracking, similar to that induced by present drop tower experiment has been reported to occur after acute joint injury, e.g., rupture of the anterior cruciate ligament (ACL)\(^23\).

**Ultrasound measurements**

The cartilage samples were imaged with a clinical high frequency (40 MHz) IVUS device (Clear view ultra, Boston Scientific, San Jose, CA, USA) (Fig. 2) before and after the mechanical injury. The ultrasound radiofrequency (RF) signal was collected from the IVUS main unit and digitized (sampling frequency 250 MHz) with a digital oscilloscope (WaveRunner 6051A, LeCroy, Chesnut Ridge, NY, USA). Subsequently, the ultrasound data was stored in the computer using custom-made LabView software (National Instruments, Austin, TX, USA). Before the measurements, the inclination between the cartilage surface and the rotation plane of the ultrasound transducer was manually adjusted with goniometers (Edmund Optics Inc., Barrington, NJ, USA) to obtain the maximum reflection from the saline–cartilage interface. When the angle between the ultrasound transducer and cartilage surface was optimal, 10 full rotations of the ultrasound transducer were recorded. During the measurement, the cartilage samples were immersed in degassed PBS. After the measurements, the samples were prepared for the histological analysis.

To calculate the absolute values of ultrasound reflection and backscattering parameters, a reference reflection was measured from a polished steel plate with a known reflection coefficient (\(R\)) (93%). The reference reflection was measured using various distances (1—4 mm, 30 measurement distances) between the ultrasound catheter and saline–steel interface. For each ultrasound measurement the reference reflection, recorded at the nearest distance to that of the reflection from the saline cartilage interface, was selected for the analysis.

**Quantitative ultrasound parameters**

Reflection coefficient (\(R\)), integrated reflection coefficient (IRC), apparent integrated backscattering (AIB) and ultrasound roughness index (URI) were determined from the ultrasound signal as described in earlier publications\(^5\)\(^6\)\(^16\)\(^17\) (Table 1). Briefly, ten full rotations of the IVUS transducer each consisting of 255 scan lines were recorded. Subsequently, one scan line perpendicular to the cartilage surface was selected from each of the 10 revolutions for the calculations of the reflection and backscattering parameters. This was achieved by selecting the scan line with the shortest distance between the ultrasound transducer and cartilage surface. \(R\), IRC and AIB were all determined from the same scan line. In the calculations of URI, 11 measurement points (five points from each side of the selected perpendicular scan line) were selected for analysis from each of the 10 revolutions of the transducer. Before the determination of URI, the trend arising from the measurement angle and natural curvature of the cartilage surface was removed from the measured surface profile by the spline fitting technique as described previously\(^8\).

Before the analysis, the ultrasound signal was filtered with a custom made digital band pass filter (fifteenth order finite impulse response, pass band 15—80 MHz). Before the calculation of IRC, the ultrasound pulse reflected from the saline–cartilage interface was Hamming windowed (length 0.2 \(\mu\)s) and before calculation of AIB, a rectangular window (length 0.48 \(\mu\)s) was selected after the IRC window.

**Light microscopy, polarized light microscopy (PLM) and digital densitometry**

To evaluate the integrity of the cartilage surfaces, the samples were imaged with a light microscope (Carl Zeiss Microlimaging Inc., GmbH 37018, Göttingen, Germany) before and after the mechanical impact. Furthermore, to quantify the histological integrity of the cartilage Mankin scoring was conducted to the Safranin O stained sections. Mankin scoring is a widely used semiquantitative scoring system in which the cartilage structure, cells, Safranin O staining (proteoglycan distribution) and tidemark integrity were scored individually and the final score value is calculated as a sum of the subscores\(^25\)\(^24\). Mankin's score values range from 0 to 14 corresponding intact and severely abnormal tissues, respectively. All the samples were scored independently by three of the authors and the final score value was calculated as an average of the three score values rounded to the nearest integer. Subsequently, mean Mankin score values were calculated for intact and injured samples. To quantify the spatial distribution of proteoglycans of the cartilage samples, digital densitometry (Leitz Ortholux II, Leitz Wetzlar, Wetzlar, Germany) of the Safranin O stained sections was conducted\(^26\)\(^23\). In order to evaluate the integrity of the cartilage collagen network PLM (Leitz Ortholux II POL, Leitz Wetzlar, Wetzlar, Germany) of the unstained sections was conducted and orientation of collagen fibrils relative to cartilage surface was calculated as a function of cartilage depth\(^26\)\(^27\).

**Statistical analysis**

Wilcoxon signed rank test was used to determine the statistical significance of difference between intact and injured cartilage in the values of ultrasound parameters, optical density, Mankin score and collagen orientations. Friedman test was used to determine significance of difference in parameter values between different injury groups. The limit of significance was set at \(P < 0.05\). All measurements were repeated three times to determine the reproducibilities of the ultrasound parameters. The reproducibilities of the ultrasound parameters were determined by calculating intraclass correlation (ICC) (SPSS 17.0, SPSS, Chicago, IL, USA).

![Fig. 2. The measurement geometry. An ultrasound catheter was fixed to the high-resolution drivers to enable accurate positioning of the transducer. Cartilage sample was fixed on the goniometers, enabling accurate control of the angle between the ultrasound transducer and cartilage surface. Cartilage samples were immersed in degassed PBS during the measurements.](image-url)
Table I  
The mathematical definitions of quantitative ultrasound parameters used in the present study.

<table>
<thead>
<tr>
<th>URI (μm)</th>
<th>R (%)</th>
<th>IRC (dB)</th>
<th>AIB (dB)</th>
</tr>
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<tbody>
<tr>
<td>URI = ( \sqrt{\frac{1}{m} \sum_{i=1}^{m} (d_i - \bar{d})^2} )</td>
<td>R = ( \frac{A}{A_{ref}} \times 100% )</td>
<td>IRC = ( \frac{1}{\Delta f} \int_{\Delta f} R_{DBC}(f) ) df</td>
<td>AIB = ( \frac{1}{\Delta f} \int_{\Delta f} R_{DBC}(f) ) df</td>
</tr>
</tbody>
</table>

The symbols used in the equations: \( m \) – number of A-mode RF signals (\( m-11 \)); \( d_i \) – distance from the transducer to the PBS-cartilage interface in \( i \)th A-mode RF signal; \( \bar{d} \) – average distance from the transducer to the cartilage surface; \( A \) – reflected peak-to-peak amplitude of the reflection occurring at cartilage surface point perpendicular to the transducer; \( A_{ref} \) – reference peak-to-peak amplitude (corrected with the steel-water \( R \)) measured from the PBS-polished steel interface at the same distance from the transducer as \( A \); \( R_{DBC}(f) \) is the frequency-dependent \( R \) (corrected with the steel-water frequency-dependent \( R \)); \( R_{DBC}(f) \) is the frequency-dependent apparent backscatter coefficient (corrected with the steel-water \( R \)); \( \Delta f \) – analyzed frequency range (-6 dB bandwidth: 30.1–45.3 MHz).

Results

Ultrasound reflection coefficients (\( R \) and \( IRC \)) decreased (\( P < 0.05 \)) after 0.12 J, 0.50 J and 0.74 J mechanical injury (Fig. 3). The values of \( AIB \) in cartilage injured by impact energies of 0.12 J and 0.74 J were lower (\( P < 0.05 \)) than those in the corresponding intact tissue (Fig. 3). The values of \( URI \) were similar in intact and injured cartilage (Fig. 3). Although ultrasound reflection was generally lower in more severely injured tissue, no statistically significant trend in ultrasound parameter values as a function of the severity of injury was detected. The reproducibility (ICC) of the ultrasound parameters was good being 0.88–0.98.

Digital densitometry detected some proteoglycan depletion in the injured cartilage as compared to the intact tissue (Table II, Fig. 4). The difference in the optical density values between the intact and injured cartilage was statistically significant only in the superficial cartilage in groups injured with 0.12 J, 0.25 J and 0.74 J impact energies (Fig. 4). No difference was detected between the intact and injured cartilage in the group injured with 0.50 J impact energy. No statistically significant trend in optical density values as a function of the severity of injury was detected. Mankin scoring of the Safranin O stained sections revealed that the cartilage injured with impact energy of 0.12 J exhibited a low-grade cartilage injury and the severity of the cartilage injury increased as the energy of the impactor increased (Table II). The mechanical injury had affected the cartilage structure and Safranin O staining subscores. However, the rupture of the cartilage surface may have caused leakage of the proteoglycans during the fixation and decalcification procedures prior to the preparation of histological sections. A statistically significant increasing trend was detected in Mankin score values as a function of the severity of cartilage injury (\( P = 0.001 \)). The collagen orientation was similar in intact and injured cartilage (Figs. 5, 6). The orientation of the collagen network as determined with PLM was similar in intact tissue as compared to injured cartilage. The orientation of the collagen fibers was approximately 70° in the deep zone and 25° in the superficial zone. This deviation from 90 and 0 orientations in deep and superficial zone may be due to incomplete maturation of the 18-month-old bovine cartilage. There was no statistically significant trend detected in collagen orientation values as a function of the severity of injury.

Light microscopic images of the cartilage surfaces, stained with Indian ink, revealed cracking of the cartilage surfaces in all the
samples injured with impact energies of 0.25–0.74 J (Fig. 6). In four out of eight cases the cartilage samples injured with an impact energy of 0.12 J showed no cracking. In all sample groups (except the 0.25 J group), the injured cartilage showed significant changes in acoustic properties.

**Discussion**

In the present study, intact and mechanically injured cartilage samples were investigated using a high frequency ultrasound device. An acute cartilage injury could be detected with quantitative ultrasound imaging. The values of ultrasound reflection parameters (R and IRC) were lower in injured cartilage than in intact tissue. However, the level of statistically significance was not reached with the 0.25 J group. The statistical non-significance relates to variations in the severity of injury induced by the drop tower and to the small number of samples in each group. Furthermore, a trend for a decrease in reflection parameters values was detected as the energy of impact increased, reflecting more severe damage to the cartilage surface (p = 0.054 and 0.062, for R and IRC respectively). The ultrasound findings were supported by the Mankin scoring that revealed increased tissue damage as the energy of the impactor increased. The mechanical injury increased the values in structure and Safranin O staining subscores. However, in cartilage with deep clefts in the surface some proteoglycans may have leaked out during chemical fixation. This could have reduced the Safranin O staining. Importantly, statistically significant difference between the intact and injured tissue in 0.12 J group was revealed in ultrasound reflection, but not in Mankin score. This indicates that ultrasound measurements might be more sensitive to minor cartilage damages than Mankin scoring.

In general, the ultrasound reflection from the cartilage surface is controlled by the cartilage surface roughness and the collagen content of the superficial cartilage. However, in the present study, no significant difference was found between the intact and injured tissue in terms of cartilage surface roughness (URI). Furthermore, PLM showed that the structure of the collagen network was nearly normal at the surface of injured cartilage. Thus, the reduction in the cartilage surface reflection might have been caused by an increase of water content at the cartilage surface. The increase in water content changes the acoustic impedance which decreases the ultrasound reflection at the saline–cartilage interface. On the other hand, ultrasound reflection has been reported to correlate with cartilage mechanical properties. The cracks in the cartilage surface, caused by the impact injury, may change the

<table>
<thead>
<tr>
<th>Energy (J)</th>
<th>Optical density</th>
<th>Mankin score</th>
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<tbody>
<tr>
<td>0.12</td>
<td>1.9 ± 0.2</td>
<td>1.3 ± 0.8</td>
</tr>
<tr>
<td>0.25</td>
<td>1.7 ± 0.2</td>
<td>1.1 ± 0.5</td>
</tr>
<tr>
<td>0.50</td>
<td>1.7 ± 0.2</td>
<td>1.4 ± 0.3</td>
</tr>
<tr>
<td>0.74</td>
<td>1.7 ± 0.2</td>
<td>1.5 ± 0.5</td>
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Injury (n = 8) Optical density 1.7 ± 0.2 1.6 ± 0.2 1.7 ± 0.2 1.4 ± 0.3
Mankin score 2.4 ± 1.2 4.1 ± 0.9* 4.8 ± 0.3* 5.5 ± 0.3*

*p < 0.05, Wilcoxon rank test.

![Fig. 4. Optical density profiles of Safranin O stained sections of intact and injured cartilage samples (mean and 95% confidence intervals, n = 8) as a function of relative depth from the cartilage surface (surface to subchondral bone). The profiles indicate spatial distribution of proteoglycans inside the cartilage. The proteoglycan concentration was slightly lower in the injured cartilage than in intact tissue. However, the difference between the intact and injured cartilage was statistically significant only in the superficial tissue. *p < 0.05, Wilcoxon signed rank test.](image)
Fig. 5. Axial orientation of the collagen fibrils relative to the cartilage surface (mean and 95% confidence intervals, $n=8$) as a function of the relative depth from the cartilage surface (surface to subchondral bone). The orientation of the collagen fibrils was similar in intact and injured cartilage.

Fig. 6. A representative ultrasound, light microscopic and histological images (Safranin O staining of proteoglycans and PLM image of collagen orientation) of intact and injured cartilage. The cartilage injury created by impact energy of 0.12 J was not visible to the naked eye but the injury could be detected by the slight decrease of proteoglycan content in superficial cartilage and the lower ultrasound reflection at the cartilage surface. Cartilage injured by dropping an impactor on a cartilage at the instant energies of 0.25, 0.50 and 0.74 J exhibited cracking of the cartilage surface and loss of proteoglycans at the superficial cartilage. The irregular surface of the injured sample could also be detected in the ultrasound images.
collagen fibril tension at the surface. This affects the mechanical properties of the superficial cartilage. It can also explain the decrease in ultrasound reflection after impact injury.

The values of URI after injury were similar to those measured in intact tissue. This result was supported by the light microscopy which showed that the injured cartilage samples exhibited cracking of the cartilage surface. However, the cartilage surface was smooth at the sites where URI was determined. Furthermore, the cracking of the cartilage surface seen in the light microscopic images of injured cartilage samples could be detected in ultrasound images. In general, the values of AIB were lower in the injured cartilage than in intact tissue. However, the difference was statistically significant only for cartilage samples injured with impact energies of 0.12 J and 0.74 J. In previous studies, increased values of AIB have been related to abnormal organization and content of collagen fibrils within the cartilage matrix. PLM demonstrated that the intrinsic collagen network was nearly normal at the measurement sites in all sample groups. This probably explains why AIB was not sensitive to indicate the severity of cartilage injury as well as why the statistical significance was reached only between the groups with the lowest and highest injuries.

The present study was conducted under laboratory conditions in vitro, enabling accurate control of the position of the ultrasound transducer. In clinical measurements, the accurate positioning of the ultrasound transducer is more challenging which may impose some limitations on the detection of the minor impact injuries in human patients. However, development of arthroscopic tools may enable more accurate controlling of the ultrasound transducer and, thus, improve the reproducibility of the measurements and increase the sensitivity of the ultrasound parameters to cartilage injuries under clinical conditions.

Currently there are few useful clinical intervention techniques for treating mild cartilage injuries, but this is an area of active research. Indeed, the development of new treatments for cartilage injuries and the planning of rehabilitation of the patient after joint injury may benefit from more accurate and sensitive assessment of cartilage injuries.

The present results support the hypothesis that an impact injury to the articular cartilage can be detected with quantitative ultrasound. As anticipated surface reflection from cartilage decreased after impact injury. However, no systematic changes were detected in ultrasound backscattering (AIB) from cartilage surface or inner structures. PLM demonstrated that the present impact loads produced only minor damage to the collagen network and, thus, URI and AIB were insensitive at revealing the impact injury.

To conclude, the present results indicate that the acute impact injury of articular cartilage can be sensitively detected with quantitative ultrasound imaging. Importantly, even submacroscopic cartilage damage could be detected. Since, the technique has been reported to be clinically applicable, it could well prove suitable for evaluating the integrity of articular cartilage after an acute impact injury, e.g., after a tear in the ACL. However, the sensitivity of the ultrasound technique at detecting acute impact injuries of articular cartilage during arthroscopy is still unclear and further clinical studies will be needed.

Author contributions

Authors Töyräs, Jurvelin, Tiitu, Timonen, and Virén substantially contributed to the conception and design of the study. Authors Timonen, Tyrvalinen, Tiitu and Viren contributed to sample preparation, acquisition of data, or analysis and interpretation of data. All authors participated in the writing process and approved the final version of the manuscript. Tuomas Virén (Tuomas.Viren@uef.fi) takes responsibility for the integrity of the work.

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Conflict of interests

The authors have no conflicts of interest.

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