

# Effect of Osteochondral Graft Orientation in a Biotribological Test System

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Received 16 March 2018; accepted 11 January 2019

Published online 28 January 2019 in Wiley Online Library (wileyonlinelibrary.com). DOI 10.1002/jor.24236

**ABSTRACT:** Autologous osteochondral transplantation (AOT) utilizing autografts is a widely used technique for the treatment of small-to-medium cartilage defects occurring in knee and ankle joints. The application of viable cartilage and bone ensures proper integration, early weight bearing, as well as restoration of biomechanical and biotribological properties. However, alignment of the autografts onto the defect site remains a pivotal aspect of reinstating the properties of the joint toward successful autograft integration. This is the first study to perform tests with different orientations of osteochondral grafts in a cartilage-on-cartilage test system. The objective was to estimate if there are differences between aligned and 90°-rotated grafts concerning molecular biological and biomechanical parameters. Tissue viability, assessed by XTT assay indicated lower metabolic activity in tested osteochondral grafts (aligned,  $p = 0.0148$  and 90°-rotated,  $p = 0.0760$ ) in favor of a higher anabolic gene expression (aligned,  $p = 0.0030$  and 90°-rotated, 0.0027). Tissue structure was evaluated by Safranin O histology and microscopic images of the surface. Aligned and 90°-rotated grafts revealed no apparent differences between proteoglycan content or cracks and fissures on the cartilage surface. Test medium analyzed after tribological tests for their sulfated glycosaminoglycan content revealed no differences ( $p = 0.3282$ ). During the tests, both the friction coefficient and the relative displacement between the two cartilage surfaces were measured, with no significant difference in both parameters (COF,  $p = 0.2232$  and relative displacement,  $p = 0.3185$ ). From the methods we deployed, this study can infer that there are no differences between aligned and 90°-rotated osteochondral grafts after tribological tests in the used ex vivo tissue model. © 2019 The Authors. *Journal of Orthopaedic Research*® Published by Wiley Periodicals, Inc. on behalf of Orthopaedic Research Society. *J Orthop Res* 37:583–592, 2019.

**Keywords:** mechanobiology; cartilage; synovium & osteoarthritis

Focal articular cartilage defects of the knee are a challenging condition that requires a surgical intervention to minimize further casualties. Defects on the articulating surfaces of the knee joint mainly occur due to traumatic injuries, avascular necrosis, or a joint deformity.<sup>1–3</sup> Here, different strategies are available for the treatment of full-thickness defects. Among them, currently used techniques include bone marrow stimulating methods (e.g., microfracture, drilling, abrasion arthroplasty, or spongialization), chondrocyte implantation directly (autologous chondrocyte implantation [ACI]) or in combination with biomaterials (matrix-associated chondrocyte implantation [MACI]). Also, intra-articular injection of lubricants (e.g., hyaluronan), as well as the implantation of single or multiple autologous grafts (mosaicplasty), are used as treatment options.<sup>1,4–6</sup>

The latter being predominantly used for small-to-medium defects, wherein the number of autologous

grafts, which can be isolated from a non-weight bearing zone of the femoral condyle, draws its limitation along donor-site morbidity. Nevertheless, osteochondral autologous transplantation is a single-step procedure in which a vital hyaline cartilaginous tissue and subchondral bone are transplanted to a primed defect.<sup>3</sup> Also, allografts yielded 12–24 h after a donor's death can be stored fresh or cryopreserved and transplanted later. In this procedure, one of the main advantages of implying osteochondral allografts is the presence of chondrocytes, which are still metabolically active.<sup>7</sup> Furthermore, osteochondral transplantation, either autologous or allogeneic, has the benefit of providing both viable hyaline cartilage and underlying bone, allowing good bony integration and early weight bearing to the joint.<sup>8</sup> Consequently, a congruent articular cartilage surface similar to that of the natural joint can be obtained which subsequently restores the biomechanical and tribological properties of the joint.<sup>2,9</sup>

In this context, tribological principles play a significant role and have gained attention over the last decade in elucidating how natural synovial joints function or fail and provide insights into different design principle criteria.<sup>10</sup> The moving contacts between the surfaces are mandatory to produce high fluid pressurization, as well as a low coefficient of friction from a tribological point of view.<sup>11</sup> Important aspects which can affect measurements are friction, wear, and lubrication. Additional aspects include debondment products of implants or from the cartilage

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Grant sponsor: Austrian COMET-Program; Grant sponsor: Project XTribology; Grant number: 849109; Grant sponsor: NO Forschungs- und Bildungsges.m.b.H. (NFB) and the provincial government of Lower Austria; Grant number: LSC14-015.

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itself, which subsequently can lead to inflammatory responses.<sup>10</sup> Numerous studies have used pin-on-disc or flat-on-flat tribometers for distinctive basic tribological models. In the latter cases, the biological cartilage–cartilage interface of a joint is replaced by an interface of cartilage against glass, metal, ceramic, or another biomaterial. However, this setup neglects the natural response of the tissue to movement and load against a soft tissue surface.<sup>10,12–14</sup> Other *in vitro* studies investigating tribological characteristics of different hydrogels were limited due to relative usage of small sample size with articulation against articular cartilage.<sup>13</sup> Other systems involving cartilage-on-cartilage systems compared only friction and lubrication of the chondroplastic materials.<sup>15</sup>

Considering the three-dimensional structure of articular cartilage and the direction of collagen fibrils responsible for its unique mechanical properties, it likely seems that the orientation of the osteochondral grafts plays a decisive role. However, the orientation of osteochondral grafts and its influence on contact pressure and chondrocyte viability has not been studied yet in a biological cartilage-on-cartilage test system. Here, *in vitro* models should represent the anatomical and physiological conditions tangibly, wherein tribological test systems could support to understand better and mimic these conditions. They can serve as a strategic preclinical test platform to evaluate treatment options and provide relevant information on the performance and suitability of implant materials.<sup>13</sup>

This study aimed to investigate the effects of orientation of osteochondral grafts in a well-established test system concerning tribological and biological outcome measures *ex vivo*. In our experimental setup, two cartilaginous surfaces were moved against each other. We hypothesized that 90°-rotated grafts could have increased surface damage, higher coefficient of friction and decreased cartilage-specific parameters (e.g., metabolic activity, gene expression) in comparison to aligned osteochondral grafts in a cartilage-on-cartilage tribological test system.

## METHODS

### Specimen Preparation

Eight bovine knees were obtained from 18- to 20-months-old slaughtered cows. Under aseptic conditions, osteochondral grafts were harvested from the medial femoral condyle using a Single-Use OATS punch (Arthrex Inc., Naples). Osteochondral grafts were marked in order to describe their orientation within the joint surface. A sterile pen was used to mark them on the anterior side of the osteochondral grafts (see Figure 2). Each knee yielded 12–16 osteochondral grafts (8 mm diameter; 15 mm height). The osteochondral grafts were washed for 2 h in phosphate buffered saline (PBS, Sigma–Aldrich Chemie GmbH, Steinheim, Germany) at 37°C to remove loose bone particles and fatty tissue. The samples were then cut to 8 mm height with a custom-made cartilage holder. Subsequently, the osteochondral samples were cultivated in a growth medium (GIBCO DMEM/F12 GlutaMAX-I,

Invitrogen, LifeTech Austria, Vienna, Austria) with antibiotics (penicillin 200 U/ml; streptomycin 0.2 mg/ml) and Amphotericin B 2.5 µg/ml (Sigma–Aldrich Chemie GmbH) for 7 days. This step was introduced to overcome a decrease in metabolic activity and equilibrate the *ex vivo* environment after the harvesting procedure.<sup>6</sup> After seven days of incubation, tribological tests for each animal were performed within the next two days at 39°C. During the testing time (2 h), the untested group was also kept at 39°C. Before and after testing samples were held at 4°C until both tested and untested osteochondral grafts were analyzed on Day 10.

### Biotribological Test System

A test system with a specially designed sample holder for this study is shown in Figure 1. It performs a reciprocal sliding movement between the osteochondral grafts. These grafts are submerged in a test fluid (in this case PBS) to mimic the conditions in the knee joint. The test setup itself encloses the sample holder to ensure sterile conditions throughout the testing process that the biological samples are not exposed to external influences and may cause artifacts which may interfere in the process of further analysis.

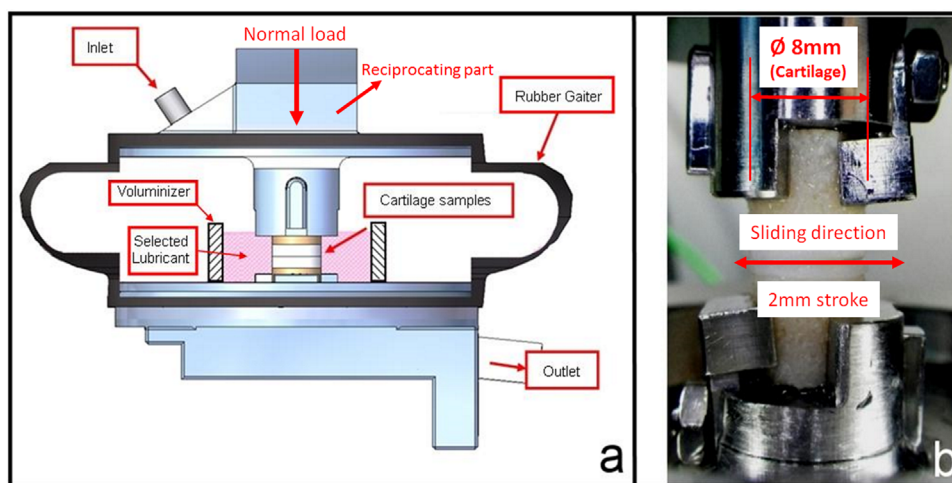
The applied load was 180 N and, considering the contact area of 50.26 mm<sup>2</sup> for ø8 mm cartilage samples, an initial estimated average pressure value of 3.57 MPa was achieved. The plane was assumed flat for all tested cartilage samples. The created tribo-model matches with contact pressure measurements for the tibiofemoral compartment of a human knee under the normal load of body weight with 0° flexion.<sup>16</sup> To mimic loading-unloading conditions of the knee during walking, the system was loaded for 10 min and then unloaded for another 10 min for a total test period of two hours with a stroke of 2 mm and a frequency of 1 Hz.

Samples stored for transportation purposes at 4°C were equilibrated to room temperature for half an hour, followed by another half an hour in the specimen holder, and were submerged in PBS at a temperature of 39°C (internal bovine body temperature).

The tribological installation was a pin-on-pin setting with two different arrangements. In one group, the samples were aligned in the same orientation as they were in the joint. The orientation was anterior to posterior in both the upper and the lower osteochondral grafts (group “aligned,” see Figure 2a). In the second group of samples, the upper osteochondral graft was rotated 90° in a clockwise direction (“90°-rotated,” see Figure 2b), whereby the orientation relative to the lower osteochondral graft changed. This arrangement should simulate implantation of an osteochondral graft during osteochondral transfer perpendicular to the anterior-posterior axis of the joint.

### Metabolic Activity

For the measurement of the metabolic activity of chondrocytes within the tissue, an XTT based *ex vivo* toxicology assay kit (Sigma–Aldrich Chemie GmbH) was used according to the manufacturer’s instructions. Cartilage was cut from the osteochondral grafts with a scalpel and divided longitudinally into two parts for XTT assay and RNA isolation. The cartilage was minced into smaller fragments onto a 24-well plate. Tissue weight for each sample was determined, and then the tissue was incubated in the XTT solution (1 ml medium, 490 µl XTT reagent, and 10 µl activation reagent) for 4 h at 37°C in 5% (v/v) CO<sub>2</sub> in the air. After incubation,



**Figure 1.** (a) Schematic of the designed test setup with specimen holder; in- and outlet could be used for a continuous medium exchange or to introduce additives to the system and observe their effects online, but it is not relevant for this experimental setup; (b) image of cartilage-against-cartilage contact in the tribo-test; the upper part of the tribometer is reciprocating during the tests.

the XTT solution was removed and retained. Tetrazolium product was extracted from the tissue using 0.5 ml dimethyl sulfoxide (DMSO), and the remaining tissue was incubated for 1 h at room temperature under continual agitation. Then the XTT and DMSO solutions were pooled, and the absorbance was measured at 492 nm and background wavelength at 690 nm as triplicates in a 96-well plate using a multi-mode microplate reader (Synergy™ 2, Winooski, Vermont) with the software Gen 5. Absorbance was normalized to the wet weight of the tissue.

#### RNA Isolation

The other half of the cartilage tissue retrieved from the osteochondral grafts was stored in RNAlater™ (Sigma-Aldrich, St. Louis, MI) at 4°C up to one week. After storage, the cartilage was minced into smaller fragments and transferred in tubes containing ceramic beads (MagNA Lyser Green Beads, Roche Diagnostics, Basel, Switzerland) with 300  $\mu$ l lysis buffer (10  $\mu$ l  $\beta$ -mercaptoethanol + 290  $\mu$ l RLT [from Fibrous Tissue Kit, Qiagen, Hilden, Germany]). Until RNA isolation, the tube was stored in liquid nitrogen. For RNA isolation, the tube was thawed and transferred to the

MagNA Lyser (Roche Diagnostics) for homogenization of the cartilage tissue. The homogenization step (6,500 rpm, 20 s) was repeated four times with a 2-min cooling phase after each step. According to the manufacturer's instruction, every sample was then incubated with 20  $\mu$ l Proteinase K (from Fibrous Tissue Kit) for 30 min for a higher yield. RNA was eluted in 30  $\mu$ l and stored at  $-80^{\circ}\text{C}$  until cDNA synthesis.

#### Gene Expression Analysis

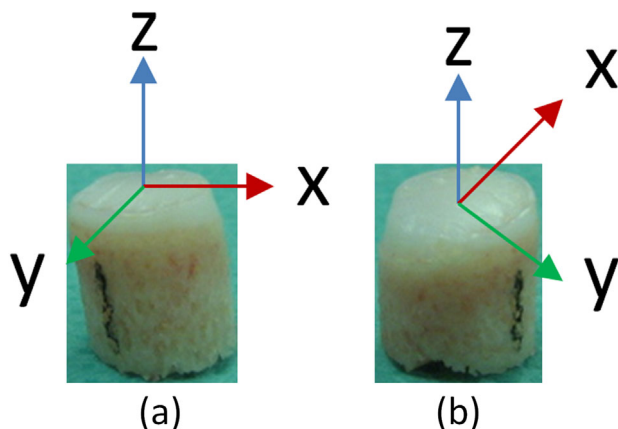
Gene expression analysis was carried out as previously described.<sup>17</sup> Briefly, cDNA synthesis was performed using Transcriptor First Strand cDNA Synthesis Kit (Roche, Basel, Switzerland). Additionally, RNA from bacteriophage MS2 was added to stabilize the isolated RNA during cDNA synthesis. Real-time quantitative polymerase chain reaction (RTqPCR) was performed in triplicate using the LightCycler® 96 from Roche. In total, four genes—Collagen type 2 (COL2A1), Aggrecan (ACAN), Matrix Metalloproteinase-1 (MMP1), and Matrix Metalloproteinase-13 (MMP13)—were analyzed, while Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as housekeeping gene.

#### Histology

For histological analysis, osteochondral grafts were fixed in 4% buffered formaldehyde solution (VWR, Radnor, PA) for up to 1 week and decalcified under constant agitation using Osteosoft solution (Merck, Burlington, MA). After decalcification (duration of 4–6 weeks), the osteochondral grafts were embedded in Tissue-Tek® OCT (Optimal Cutting Temperature, VWR, Radnor, PA) and stored at  $-80^{\circ}\text{C}$ . Sectioning was done using the CryoStar™ NX70 Cryostat (Thermo Fischer Scientific, Waltham, MA), with  $-25^{\circ}\text{C}$  for the knife temperature and  $-20^{\circ}\text{C}$  for the chamber temperature. A 6  $\mu$ m sections were obtained and processed for Safranin O staining. Images were taken with a Leica microscope DM-1000 and processed using the Leica Manager software (Leica, Wetzlar, Germany).

#### Alicone 3D Microscope

Alicona 3D microscope was used to optically analyze cartilage surface before and after the test to reveal determinant surface roughness parameters for the tribological performance of cartilage tissues. PBS was frequently added every



**Figure 2.** (a) Visualization of osteochondral graft sample where the x-axis is aligned with walking direction; (b) 90°-rotated (around z-axis) specimen on top.

5 min to prevent the cartilage surface from drying out. First, widely applied roughness parameters, such as the average (Ra), root mean square roughness (Rq), skewness (Rsk), and kurtosis (Rku) are considered. The initial image of the cartilage (Figure 3a) has been cropped to center 4 mm diameter zone to avoid impaction effects on the cartilage surface analysis (Figure 3b). Then, the image was leveled (Figure 3c) and form removed (Figure 3d) to reduce location related inconsistency of the surface profile. This means that a form component must be removed before any other metrological operation. When the form is just a line segment or a plane, this operation is called leveling. In a nonplanar form, it is called form removal. As surface texture parameters depend on a reference (mean line or mean plane) used in the detection of peaks and valleys, or on the calculation of mean values and moments, form removal and leveling make it possible to define this reference. In our case, due to the location of the sample derived or the punching process, the surface could be tilted or concave. In order to remove the tilt, the leveling process is applied to the outcome of the 3D microscope image, as the tilt refers to a line or plane adjustment. To further remove the concave geometry, 2nd-degree polynomial or spherical for the removal process was applied.

#### Sulfated Glycosaminoglycans (sGAG)

The quantification of sGAG was performed according to Barbosa et al.<sup>18</sup> In brief, fluid (PBS) used during tribological tests was treated overnight with 25 U/ml proteinase K (Sigma–Aldrich) at 56°C. After inactivation of the enzyme (90°C, 10 min), the fluid was collected in ultra-free filter reaction tubes of 0.1 µm pore size (Millipore, Burlington,

MA) and centrifuged (12,000g, 4 min, room temperature). One milliliter of a 1.9-dimethyl-methylene blue solution (DMMB) was added to 100 µl filtrate and vigorously mixed to allow the formation of complexes of DMMB and sGAG in the sample. The complexes were pelleted via centrifugation (12,000g, 10 min, room temperature) and subsequently dissolved in decomplexation solution. After 30 min of shaking, the absorbance was measured at 656 nm photometrically using an Ultrospec 3300 pro photometer (Amersham Bioscience plc, Amersham, UK). The sGAG amount was calculated from a standard curve with shark chondroitin sulfate (Sigma–Aldrich). The measurement for each aligned or 90°-rotated test of osteochondral grafts was performed in duplicate.

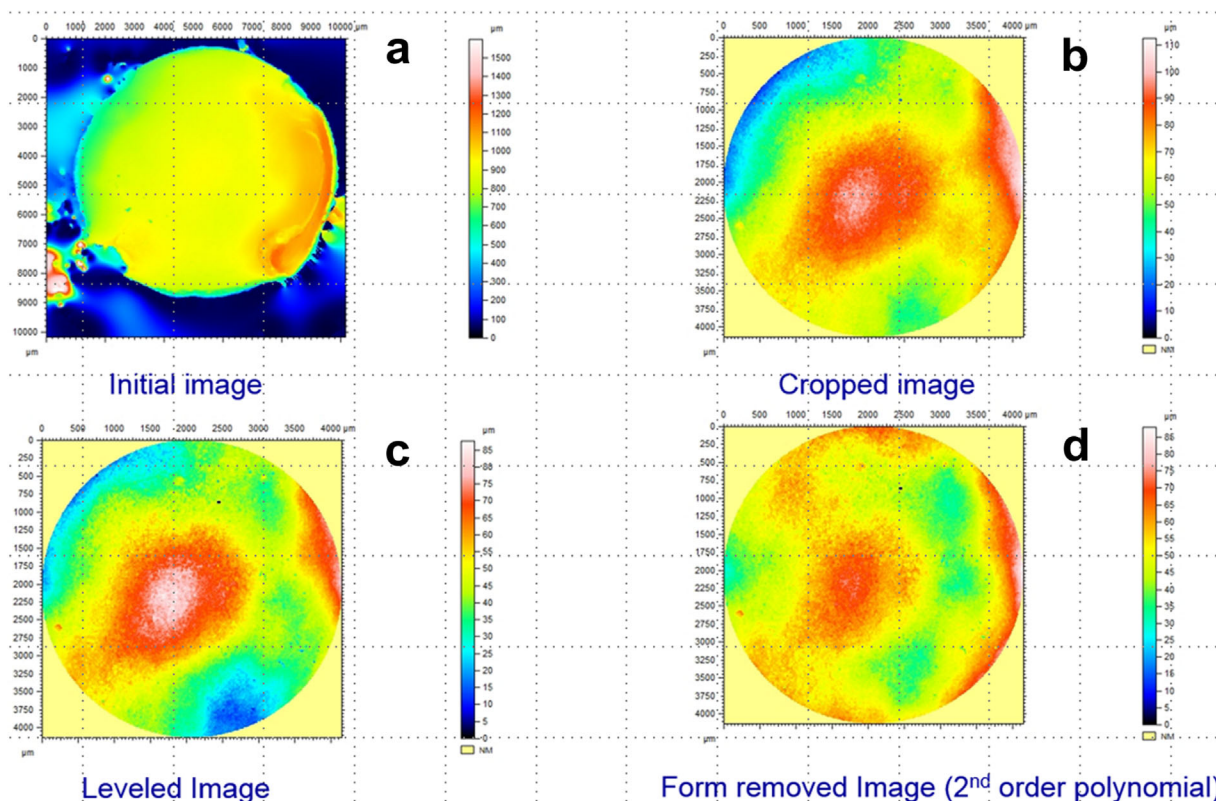
#### Statistical Analysis

All data are expressed as means ± standard deviations (S.D.) in a box plot. Non-parametric Mann–Whitney U-test was used to make comparisons between paired data, and multiple comparisons were performed by non-parametric Kruskal–Wallis test followed by Dunn's posthoc test. Statistical significance was set at  $p < 0.05$ . All statistical analysis was performed using GraphPad Prism Software (Graphpad Prism Software Inc., San Diego, CA).

## RESULTS

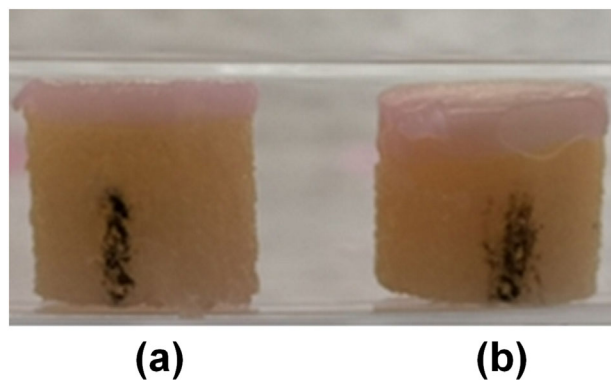
### Osteochondral Grafts

Punched out osteochondral grafts for tribological tests had a symmetrical flat surface in almost every sample used for the experiments (Figure 4a). Asymmetric grafts (Figure 4b), which were not possible to circumvent, were



**Figure 3.** (a) Initial image of cartilage surface; (b) surface after cropping to center location; (c) leveled surface image; (d) form removed final version of surface to be analyzed.





**Figure 4.** (a) Example for a used osteochondral graft for testing showing a symmetrical flat surface; (b) asymmetric graft used for control (Day 0) and untested groups.

used for control (Day 0) and the untested group as the flatness is not a critical factor as for the ones used within the tribological test system.

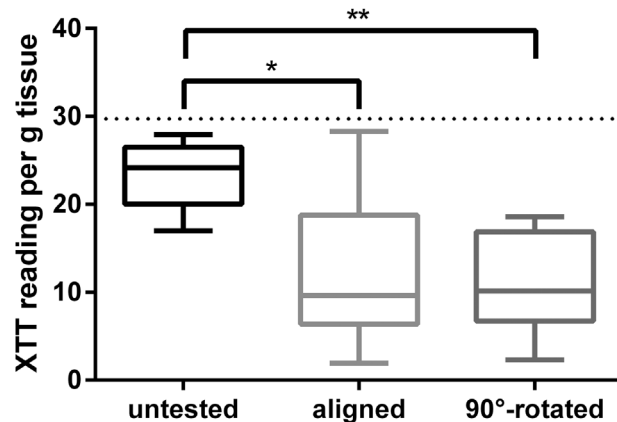
#### Metabolic Activity of the Cells

The metabolic activity of chondrocytes in osteochondral grafts showed a constant value on Day 0 (dotted line) within all animals (Figure 5), but on Day 10 the metabolic activity exhibited a significant difference ( $p < 0.05$ ) between untested osteochondral grafts and the two test groups (aligned,  $p = 0.0148$  and  $90^\circ$ -rotated,  $p = 0.0076$ ). Comparisons for metabolic activity between tested osteochondral grafts in aligned versus  $90^\circ$ -rotated orientation indicated no significant differences ( $p > 0.9999$ ). Nevertheless, in both groups, the absorption per gram of tissue was on a much lower level compared to Day 0 or on Day 10 in the untested group.

#### Expression of Anabolic and Catabolic Cartilage-Specific Genes

For the analysis of gene expression, specific bovine primers were designed and tested successfully regarding optimal temperature values for primer annealing. After 10 days, the anabolic marker COL2A1 of the untested group decreased by fourfolds from Day 0 after harvesting of the osteochondral grafts.

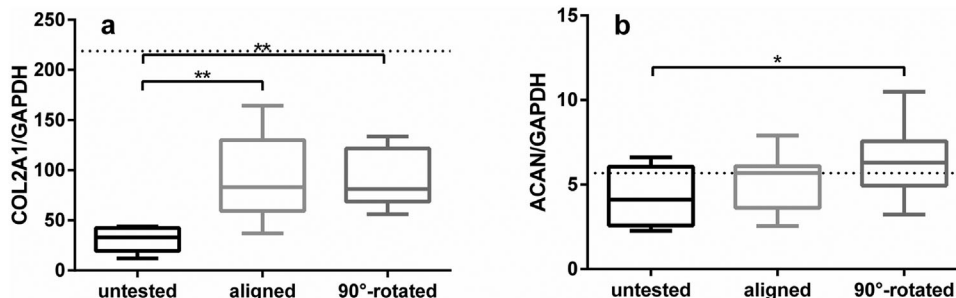
In comparison, the results of the tested osteochondral grafts were significantly higher (aligned,  $p = 0.0030$  and  $90^\circ$ -rotated,  $p = 0.0027$ ) than the



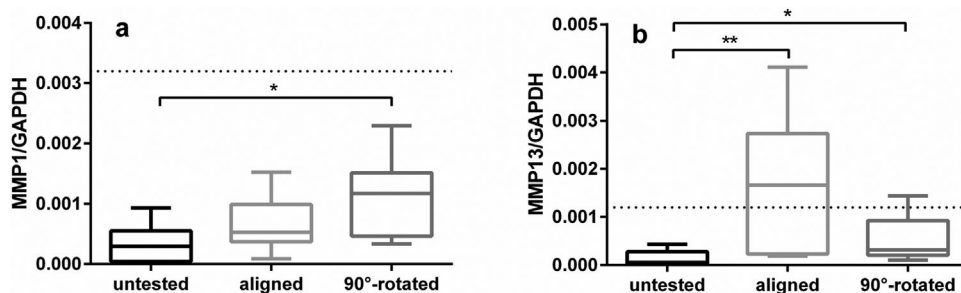
**Figure 5.** Metabolic activity of osteochondral grafts in untested and tested (aligned,  $90^\circ$ -rotated) conditions after 10 days of incubation with a comparison to day 0 (dotted line).

untested group on day 10 but showed no differences ( $p = 0.9314$ ) concerning aligned or  $90^\circ$ -rotated orientation of the grafts during testing (Figure 6a). ACAN, another anabolic marker for gene expression, did not show this dramatic reduction from Day 0 to 10 in the untested group compared to COL2A1, but also had a slightly higher (aligned,  $p = 0.4807$ ) and significant difference ( $90^\circ$ -rotated,  $p = 0.0274$ ) value. Between the two tested groups within the tribological test system, no significant difference ( $p = 0.1615$ ) was shown (Figure 6b).

However, osteochondral grafts which were tested in a  $90^\circ$ -rotated orientation had a slightly higher expression for the gene ACAN. In Figure 7, the catabolic genes MMP1 and MMP13, both of which are essential in the breakdown of interstitial collagens; for example, types I, II, and III were less expressed on day 10 in the untested group compared to Day 0. MMP1 gene expression increased significantly ( $p = 0.0104$ ) between untested and  $90^\circ$ -rotated grafts (Figure 7a) and showed no significance between untested and aligned ( $p = 0.1206$ ) or aligned and  $90^\circ$ -rotated ( $p = 0.2319$ ) groups. MMP13 showed a significant difference in both aligned and  $90^\circ$ -rotated osteochondral grafts (aligned,  $p = 0.0070$  and  $90^\circ$ -rotated,  $p = 0.0205$ ) compared to the untested group, but no significance ( $p = 0.1520$ ) within the tested groups (Figure 7b).



**Figure 6.** Gene expression of anabolic cartilage-specific genes. (a) COL2A1; and (b) ACAN expression in untested and tested osteochondral grafts after 10 days of incubation. In both graphs, Day 0 is shown as a dotted line.



**Figure 7.** Gene expression of catabolic cartilage-specific genes. (a) MMP1 and (b) MMP13 expression in untested and tested osteochondral grafts after 10 days of incubation. In both graphs, Day 0 is shown as a dotted line.

### Histology

For histological analysis, Safranin O staining was performed as shown in Figure 8. As an additional statement to microscope images of the surface, the histological evaluation was also used to find any differences (e.g., cracks or fissures) between the aligned and 90°-rotated osteochondral grafts.

Images of the untested control tissue sections stained with Safranin O revealed different strengths in the intensity of the staining for grafts from different points of withdrawal of the used medial cartilage. This was also shown in the osteochondral grafts tested in the tribological test system. So, no inference concerning proteoglycan content between the untested, aligned, and 90°-rotated group was detected.

### Microscope Images

Before tribological tests, the microscopic images of the surfaces of the aligned and the 90°-rotated groups showed no cracks or fissures, but height differences in the starting material could be visualized by the used method (Figure 9). In these samples, there was a deviation of up to 100 microns. However, this was not included in our observations, because we focused more on cracks and fissures on the surface, which were achieved from the applied strain under physiological conditions (3.57 MPa). Our results demonstrate that from the specified applied strain, cracks and fissures occurred but no differences in depletion of the proteoglycan content between loaded (aligned and 90°-rotated) and untested grafts were seen. The severity of these damages ranges from deep trenches (more than

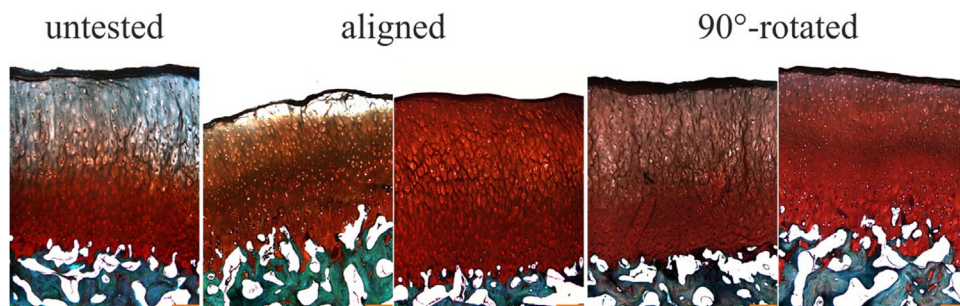
200  $\mu\text{m}$  deep) to small superficial cracks (up to 50  $\mu\text{m}$ ). Also, there were osteochondral grafts that did not show any damage in aligned and 90°-rotated orientation. This finding makes it impossible to define a difference in cartilage surface area concerning aligned and 90°-rotated osteochondral grafts tested in the tribological test system.

### Coefficient of Friction (COF) and Relative Displacement

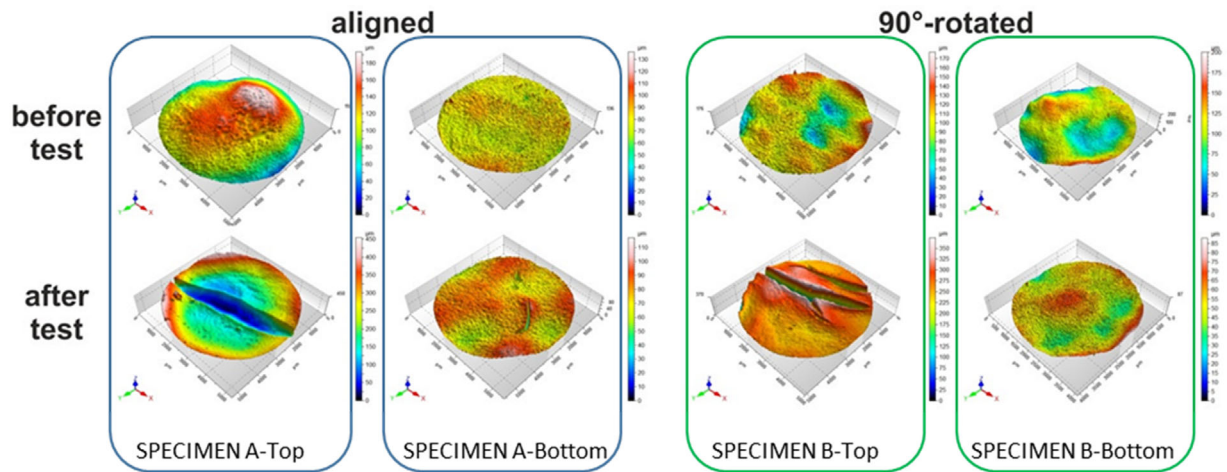
During tribological tests, COF values in aligned osteochondral grafts varied between 0.012 and 0.016 during each test cycle (Figure 10). In comparison, 90°-rotated grafts showed similar results at the end of each cycle with no significant difference ( $p = 0.2232$ ). The range was from 0.012 to 0.018 (Figure 11). Also, increasing relative displacement between the top and bottom holders, during the 10-min stimulation phase, was observed in both test groups. The values in aligned orientated osteochondral grafts were around 900  $\mu\text{m}$  (Figure 10) and so much lower than in 90°-rotated grafts, where the values reached up to 1300  $\mu\text{m}$  (Figure 11).

### sGAG in the Supernatant After Testing

In the aligned and the 90°-rotated osteochondral grafts, the test fluids (PBS) after tribological tests were collected and measured for their content of released or abraded sulfated glycosaminoglycans. There was no significant effect in sGAG content between the orientations of osteochondral grafts in our test setup as both groups in Figure 12 showed similar values in the fluid after tribological tests.



**Figure 8.** Sections of cartilage tissue from untested and tested (aligned, 90°-rotated) osteochondral grafts on Day 10 stained with Safranin O; scale bar 250  $\mu\text{m}$ .



**Figure 9.** Output of 3D microscope representing major changes on the surface structure following tribological tests for aligned and 90°-rotated samples.

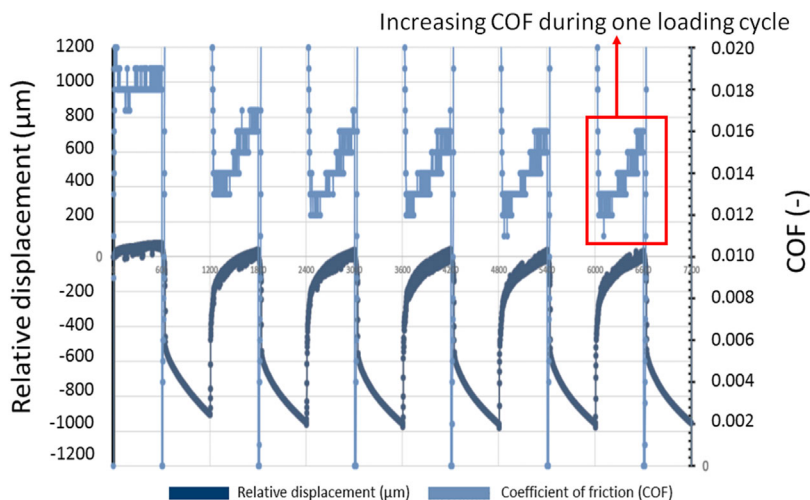
## DISCUSSION

The objective of the current study was to investigate possible differences between aligned and 90°-rotated bovine osteochondral grafts in an established cartilage-on-cartilage (COC) biotribological test system. Bovine osteochondral grafts from the medial femoral condyle were investigated due to experimental reproducibility and availability. We could demonstrate that both aligned and 90°-rotated grafts showed higher expression of anabolic makers and decreased metabolic activity compared to controls. There were no differences between the two treatment groups for the coefficient of friction, proteoglycan content, and sulfated glycosaminoglycan (sGAG) release in the test fluid. Our results suggest that the orientation of osteochondral grafts regarding rotation relative to the joint axis might not have an impact on cartilage wear and metabolic outcome measures. While alignment of the graft and proper restoration of the articular surface have been shown to be crucial during autologous osteochondral transplantation, our findings indicate that there are no differences in the

osteocondral graft orientation in the used ex vivo tissue model.

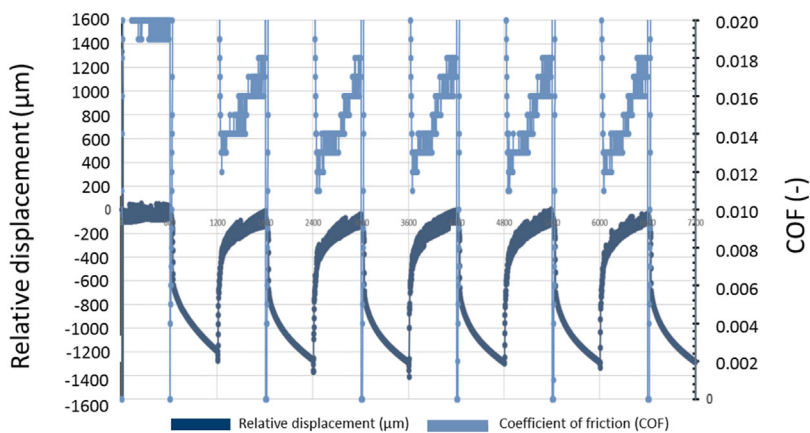
The use of a COC biotribological test system has been used as it more closely reflects physiological conditions compared to other counterpart materials like metal or glass. Similarly, metal-on-cartilage test systems led to an increase in apoptotic chondrocytes compared to the COC test systems. However, these differences can occur due to different loading magnitudes and loading rates, which in turn result in differences between structural damage of the articular cartilage.<sup>19</sup> To ensure stable contact between cartilage surfaces, a normal pressure of 180 N exerting a pressure of 3.57 MPa on the cartilage surface of the osteochondral grafts was deployed in our experimental setup. Consequently, the tribological test system is very similar to the pressure measurements of the tibial-femoral compartment of a human knee joint under normal body pressure.<sup>16</sup>

Additionally, during the testing period of 2 hours, the coefficient of friction (COF) and relative displacement was also recorded as indicators of mechanical



**Figure 10.** Coefficient of friction and relative displacement values versus time for aligned samples.





**Figure 11.** Coefficient of friction and relative displacement values versus time for 90°-rotated samples.

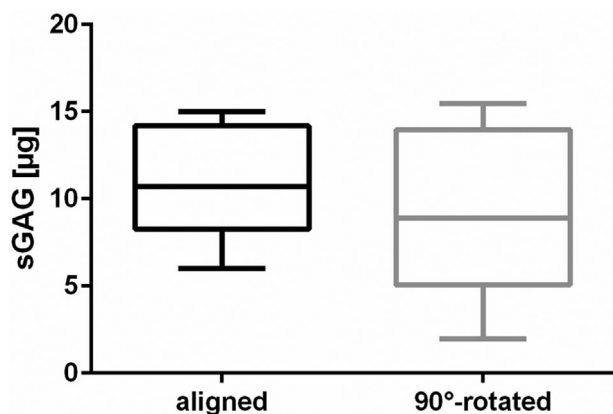
stress. In healthy synovial joints, the COF values are approximately around 0.005.<sup>20</sup> This low value could not be reached during the tests in our biotribological test system, as PBS was used instead of synovial fluid as a cartilage-surrounding fluid. The COF values during the tests varied between 0.012 and 0.018, whereas the aligned grafts reported slightly lower values. The increase from 0.012 to 0.016 (aligned) or 0.018 (90°-rotated) during a test cycle could occur from abnormal loading conditions that lead to extensive creep and exudation of the tissue. Here, increasing relative displacement values from 900µm to 1300µm during the loading cycle may hint at the conformity of cartilage.

For the harvesting of osteochondral grafts, different methods being used in clinics were available. In our study, however, we used single-Use OATS as it is the most common harvesting device. Besides, it was shown that cell viability around the edges of osteochondral grafts is best conserved by this method compared to the other punching or rotating techniques.<sup>1</sup> Before and after the tribological tests, microscopic images were taken and additionally processed to obtain a 3D topographical image of the cartilage surface. Various areas on the cartilage surface showed ruptures which had a depth of up to 200 µm in both tested groups. The shape of the ruptures and the formation of surface

fissures show similar properties to those reported by other studies.<sup>21,22</sup> Concerning the evaluation of the cartilage surface, histology was additionally used to show modifications of the surface and the proteoglycan content of the cartilage itself. Tested osteochondral grafts were compared with a control group after a total incubation time of 10 days. Differences between the groups could not be assessed, as also indicated by Tekari et al., as over the whole cartilage surface proteoglycan content, but not gene expression patterns, were varying.<sup>23</sup> After harvesting osteochondral grafts, the locations within the donor site were numbered on taken pictures to always use grafts from more or less the exact location in all the animals used. This repetitive process was not always possible as cuts and osteochondral defects occurred on the donor site and grafts had to be taken out from different areas on the medial condyle.

The release of sulfated glycosaminoglycans (sGAGs) into the culture medium in organ cultures with full-depth articular cartilage is used to detect or quantify tissue degradation.<sup>6,14</sup> In our study, the release of the sGAGs was used as a parameter to determine the breakdown of the cartilage concerning different orientations of the OC grafts. Since the measurements of the sGAGs for aligned and 90°-rotated grafts reached nearly identical levels, it was not possible to make any statements regarding differences in the release of the two test conditions. However, since damages in the form of cracks and fissures occurred under both conditions, the result was not unexpected. Another possibility that could have influenced the sGAG value is the washing out of proteoglycans from the peripheral areas of the extracellular matrix as a result of the missing lateral constraint as well as the disruption of non-covalent interactions between aggrecan and long chains of hyaluronic acid molecules in cartilage.<sup>6</sup>

Besides the analysis of tissue parameters, the chondrocytes within the cartilage were also considered. As the mitochondrial activity of chondrocytes within osteochondral grafts decreases after harvesting procedure and stabilizes after approximately 6 days,<sup>6</sup> the grafts were first incubated in culture medium for 7 days to ensure stabilized mitochondrial activity,



**Figure 12.** Release of sulfated glycosaminoglycans into the test fluid by osteochondral grafts after tribological tests on Day 10.



before tribological tests were performed. After these tests, both groups (aligned and 90°-rotated) showed the same level of activity, which is much lower compared to the untested group. This phenomenon could be the result of testing itself, as chondrocytes were possibly detached from the superficial layer<sup>24</sup> due to applied surface strain and hence a reduced cell count occurs in the cartilage tissue. Also, it could be possible that cells reduce metabolic activity in favor of performing more synthesis of, for example, collagen 2 or aggrecan. The latter case seems sensible, primarily because gene expression of collagen 2 in tested osteochondral grafts significantly increased. Aggrecan tends to increase in aligned grafts, but only the comparison between the untested group and 90°-rotated grafts is significant. However, no significant difference between the two test groups is noted. Additionally, to the two cartilage-specific genes collagen 2 and aggrecan, the matrix metalloproteinases MMP1 and MMP13 were analyzed for their gene expression. The significant increase in both genes could be due to inflammatory processes or tissue remodeling. As no inflammation mediators were used or introduced in this model, it is apparent that tissue remodeling initiated due to mechanical stress is happening.<sup>24,25</sup>

However, there are several limitations in this experimental setup. First, it must be considered that ex vivo culture often limits oxygen and nutrient supply, usually occurring under physiological conditions. Second, osteochondral grafts were harvested from 18 to 24-month-old animals. At this age, cattle are considered young but are expected to reach skeletal maturity.<sup>26</sup> Anyway, no direct link to mature human tissue concerning biological and biomechanical properties can be done.

Furthermore, our model only shows short-term effects on cartilage tissue and does not include other components of a joint. Influences of synovium, synovial fluid, meniscus, or subchondral bone are not considered. As the last point, the analysis of sGAG in the test medium is an important marker of wear and is associated with histology and metabolism.

Nevertheless, other clinically relevant markers such as Hydroxyproline (HYP) or cartilage oligomeric matrix protein (COMP) could be investigated. In particular, COMP is sensitive to mechanical stress, while HYP is used to detect collagen fragments as a structural marker of injury.<sup>27</sup> Overall, it has to be considered that all these experiments were performed ex vivo. The levels of different parameters analyzed could look different in vivo.

The alignment of osteochondral grafts in a cartilage-on-cartilage biotribological test system has not been studied extensively yet. Only a simulation study on osteochondral grafting was done, which showed effects between differently aligned osteochondral grafts after removal from an unloaded area and implantation in a loaded zone. However, this was only shown in computer simulation models and not in vivo.<sup>28</sup> Therefore, our biotribological system could

provide further insights, as biological samples are used, and the system uses pressure loads similar to those in the human knee joint. Nevertheless, no definite tendency or effect was found in molecular biological and biomechanical parameters with the used methods, so we can conclude that no differences could be shown in this ex vivo tissue model.

## AUTHOR CONTRIBUTIONS

All authors contributed extensively to the work presented in this paper. C.B. contributed in study plan, harvesting grafts, biological analysis, data analysis, and wrote the manuscript. H.G. contributed in study plan, tribological tests, microscopic images, and wrote the manuscript. E.N.M. contributed in harvesting grafts, biological analysis, and discussed the results. V.J. contributed in discussing the results, data analysis, proofreading, and commented on the manuscript. I.T. and T.K. discussed the results and commented on the manuscript. C.S. contributed in harvesting grafts, biological analysis, discussion of the results, and commented on the manuscript. F.F. supervised tribological tests and acquired funding. S.N. supervised biological evaluations, acquired funding, came up with the idea for this study, discussed results, and commented on the manuscript.

## ACKNOWLEDGMENTS

This work was funded by NÖ Forschungs-und Bildungsges.-m.b.H. (NFB) and the provincial government of Lower Austria through the Life Science Calls (Project ID: LSC14-015) and has been carried out within Danube-University Krems—University for Continuing Education in cooperation with the Austrian Excellence Centre for Tribology (AC2T research GmbH) and Landesklinikum Baden-Mödling supporting this study. The authors also gratefully acknowledge funds for parts of this work from the Austrian COMET-Program (project XTribology, no. 849109) via the Austrian Research Promotion Agency (FFG) and the Province of Lower Austria, Vorarlberg, and Wien.

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