Is There a Scientific Rationale for the Refixation of Delaminated Chondral Flaps in Femoroacetabular Impingement? A Laboratory Study

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Running Title: Refixation of Delaminated Chondral Flaps

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Abstract

Background Debonding of the acetabular cartilage is a characteristic type of hip damage found in cam type femoroacetabular impingement, which remains a treatment challenge. In addition to resection, refixation of these flaps using fibrin sealants has been recently suggested. However, there is only limited evidence available that the proposed refixation method results in sufficient viable cartilage formation to ensure long-term grafting of the flap and restored tissue function.

Questions/purposes To determine flap tissue characteristics that would justify refixation of delaminated chondral flaps with a fibrin sealant, we characterized (1) the extracellular matrix (ECM) of chondral flaps in terms of chondrocyte viability and distribution of ECM components and (2) the chondrogenic potential of resident cells to migrate into fibrin and produce a cartilaginous matrix.

Methods Ten acetabular chondral flaps and three non-delaminated control cartilage samples were resected during surgery. Chondrocyte viability was quantified using a live-dead assay. To assess the ECM, histological staining of glycosaminoglycans, collagen II and collagen I allowed the qualitative study of their distribution. Chondrocyte ability to migrate out of the ECM was tested by encapsulating minced flap cartilage in fibrin gels and semi-quantitatively assessing the projected area of the gel covered with migrating cells. The potential of chondrocytes to produce a cartilaginous matrix was studied with a pellet assay, a standard 3-D culture system to test chondrogenesis. Positive controls were pellets of knee chondrocytes of age-matched donors, which we found in a previous study to have a good capacity to produce cartilage matrix. Statistical significance of controlled quantitative assays was determined by the Student t test with Welch's correction.

Results The proportion of viable chondrocytes in flaps was lower than in non-delaminated

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cartilage (50% ± 19% vs 76 ± 6%; p = 0.02). Histology showed a disrupted ECM in flaps as compared to non-delaminated controls, with the presence of fibrillation, a loss of glycosaminoglycan at the delaminated edge, collagen II throughout the whole thickness of the flap and some collagen I-positive area in two samples. The resident chondrocytes were able to migrate out of this disrupted ECM in all tested samples. However in pellet culture, cells isolated from the flaps showed a qualitatively lower chondrogenic potential compared with positive controls, with a clearly inhomogeneous cell and matrix distribution and an overall smaller projected area (0.4 versus 0.7 mm^2; p = 0.038).

Conclusion Despite the presence of viable chondrocytes with migration potential, the cells reside in a structurally altered ECM and have limited capacity to deposit ECM, leading us to question their capacity to produce sufficient ECM within the fibrin sealant for stable long-term attachment of such flaps.

Clinical Relevance The characterization of delaminated cartilage in cam FAI patients suggests that the refixation strategy might be adversely influenced by the low level of ECM produced by the residing cells.

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Introduction

Cartilage delamination (also referred to as a "cartilage flap") is a frequent finding of acetabular cartilage damage secondary to cam type femoroacetabular impingement (FAI) and is classified as Stage 4 damage according to Beck et al. [5]. The presence of such flaps has been documented in 37% to 85% of patients undergoing surgery [2, 4, 10, 20, 21, 31], with an increased incidence in male patients [2, 31]. Despite an increased failure rate of open surgery for patients whose hips demonstrate cartilage damage, there is no consensus on how to best treat chondral flaps. Débridement (removal) of cartilage is used to resolve symptoms and allow for regrowth of repair cartilage. Conversely, reattachment (refixation) of cartilage with a fibrin sealant would theoretically prevent migration of the femoral head in the defect and thus joint space loss. Refixation could also potentially augment the reintegration of the cartilage if viable chondrocytes secreted sufficient extracellular matrix (ECM) to strengthen the interface between the flap and the subchondral bone.

Kommentiert [TjG1]: AU: Fair to add this qualifier? ED

AU response: yes

Recently, viable cells in flap tissues were reported, although the reported viability values vary widely (from 39% to 95%) [11, 18, 35, 43]. Additional efforts have been made to characterize the matrix composition of the chondral flaps with histologic [23, 35] and biochemical [18] studies. These results have consistently highlighted the fibrocartilaginous nature of the ECM within the flaps with fibrillations and loss of glycosaminoglycan (GAG) staining. Still, none of these studies have had optimal controls, nor did they aim to demonstrate whether the residual cells could actively produce cartilaginous ECM. The production of ECM would be important to support refixation and chondral flap repair.

Therefore, we investigated flap tissue characteristics in patients with FAI that would support the refixation of delaminated chondral flaps with fibrin sealants, namely (1) the quality of the ECM of chondral flaps as measured by chondrocyte viability and distribution of ECM

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components compared with controls and (2) the chondrogenic potential of resident cells in terms of their ability to migrate into fibrin and produce a cartilaginous matrix.

Materials and Methods

Experimental Overview

This study had two main goals: First, to assess the quality of the ECM of chondral flaps (the anatomical position of such flaps is schematized in Fig. 1A ; the orientation of resected flaps with a suture is illustrated in Fig. 1B), and second, to evaluate the chondrogenic potential of the cells residing in the flaps. For the first part, to assess the quality of the flap ECM, we focused on (1) cell viability, as healthy tissue presumably contains a majority of viable cells, and (2) cartilage ECM distribution, as the function of the tissue is linked to its structure. Both tests were performed on sectioned tissue samples (Fig. 1C), either a slice of fresh tissue (for viability) or a 5µm-thick slice of tissue embedded in paraffin (for histological staining of ECM). In the second part, we investigated the chondrogenic potential of the residing cells by studying (1) their migration capacity and (2) their ability to produce cartilaginous matrix. Considering the poor stability of fibrin glue [13], attachment of the flap to the subchondral bone relies on the premise that cells residing in flaps can migrate into the fibrin and subsequently deposit matrix. The capacity of the cells to migrate out of the ECM into fibrin glue was tested on minced flap pieces embedded in fibrin gels (Fig. 1D). The capacity of the cells to produce cartilaginous matrix was assessed on cells enzymatically isolated from the flaps, expanded and cultured in pellets (Fig. 1E). Of note, the small size of the flap tissues did not allow us to perform all four assays with sections (viability, ECM composition), pieces (migration) and cells (ECM deposition) of the same piece of tissue (Table 1). This explains why the sample size is not 10 for all assays.

Sample Collection

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Kommentiert [CO2]: AU: Figures must appear in proper alphanumeric order, that is, Fig. 1C cannot appear in the text before Fig. 1A. Please reorder your figures.

Author response: Fig. 1A and Fig. 1B were not mentioned before, this was corrected by the addition lines 69-70.

Acetabular chondral flaps, resected during surgical procedures, were obtained from 10 patients (nine male and one female) who had a primary diagnosis of cam-type FAI with Grade IV cartilage lesions according to Beck et al. [5]. Patients had no history of childhood hip disease, no previous hip surgery, and were between 20 and 50 years old. The mean period of symptoms was 3.0 +/- 1.9 years, the mean Tegner activity score [38] was 5.3 +/- 1.8 and the mean calculated Tonnis grade was 0.3 +/- 0.5 (Table 2). All male patients underwent surgical hip dislocation; the female patient was treated with hip arthroscopy (Table 2). Inclusion criteria were cam-type FAI without signs of instability (lateral central edge angle < 25°, acetabular index > 10°, neck shaft angle < 135°, antetorsion < 15°, see Table 3), as measured from radiographs (Fig. 2). Patient consent was received and approved by the Ethics Commission of the Canton of Zurich (Kantonale Ethikkommission, Kanton Zürich, approval number KEK-ZH 2013-0097). The highly fibrous parts appearing to be the labrum (when the cartilage separation included the chondrolabral junction) were removed, and the remaining cartilage was minced for further tests (Fig. 1). Control, non-delaminated acetabular cartilage samples used for viability tests were obtained from patients undergoing either arthrodesis or THA for avascular necrosis (AVN) with laterally adherent cartilage. The AVN was due to a procedure performed for the diagnosis of slipped capital femoral epiphysis. Although AVN can potentially lead to secondary cartilage alterations, the intraoperative visual aspect showed the presence of areas with no obvious cartilage alterations, and this patient was therefore included in the study. Adult knee chondrocytes (used as controls for the pellet assays) were obtained from healthy-appearing (white-blueish color, no fibrillation, glistening aspect) articular cartilage pieces obtained from TKAs (approval number KEK-ZH 2017-00381), for instance when the medial condyle is affected by varus arthritis but not the lateral condyle.

hat gelöscht: All clinical information about the enrolled patients, including the duration of symptoms, the activity level …

hat gelöscht: ,

hat gelöscht: osteoarthritis degree,

hat gelöscht: no history of childhood hip disease, no previous hip surgery, and age between 20 and 50 years

hat gelöscht: .

hat gelöscht: the type of surgeries and the acetabulum and femur radiographic measurements are provided in Tables 2 and 3 as well as Fig. 2

hat gelöscht: .

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124 *Chemicals*

125 All chemicals were ordered from Sigma-Aldrich (Buchs, Switzerland) unless mentioned
126 otherwise.

127 *Chondrocyte Viability – Quantitative Measurement with Live-dead Staining*

128 Chondrocyte viability was investigated using a standard live-dead assay according to the
129 manufacturer's instructions, as previously described [27]. Viability was quantified with Fiji
130 software, calculated as the number of living cells divided by the total number of cells (live
131 and dead) and averaging three values for each patient's sample.

132 *Quality of ECM Compared with Non-delaminated Controls – Qualitative Assessment of*
133 *Matrix Components with Histology on Paraffin Sections*

134 Oriented native tissue samples were fixed overnight in 4% formaldehyde, washed in
135 phosphate-buffered saline, dehydrated, and embedded in paraffin. Five-microns thick paraffin
136 sections were deparaffinized in xylene and rehydrated in a series of ethanol baths with
137 decreasing concentrations of ethanol. Immunostaining of type I and II collagens were
138 performed as previously described [27], using products from Abcam (Cambridge, United
139 Kingdom) unless specified otherwise. Briefly, after antigen retrieval with hyaluronidase,
140 sections were blocked with 5% normal goat serum and primary antibodies, diluted in 1%
141 normal goat serum, were applied overnight at 4° C (rabbit anti-human Type II collagen
142 antibody, 600-401-104, 1:200/Type I collagen antibody, #ab138492, 1:1500). For negative
143 controls, slides were incubated without a primary antibody. Endogenous peroxidase activity
144 was quenched with 1% H_2O_2 for 20 minutes before applying the secondary antibody (goat
145 anti-rabbit IgG-HPR ab6721, 1:1500 dilution). Antibodies were detected with an Abcam
146 DAB kit, as per the manufacturer's protocol. Staining with hematoxylin and eosin (H&E),

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Alcian blue (pH 2.5) and Safranin O was performed in accordance with standard protocols. All stainings were followed by dehydration and two baths of xylene before the slides were mounted with a resin mounting media.

All samples were stained at the same time to avoid slight changes of intensity, which could result from sequential staining. The qualitative assessment was a yes/no answer to the following questions: (1) Are there fibrillations in the H&E sections? (2) Is a clear red staining visible in the Safranin O sections? (3) Is there a gradient in Safranin O with increasing intensity from surface to deeper layer, as expected in healthy cartilage? (4) Is there a positive type I and II collagen staining? All stainings were observed and described by three researchers (two blinded, one not blinded) to ensure interobserver repeatability.

Chondrogenic Potential of Flap Chondrocytes - Semi-quantitative Assessment of Chondrocyte Migration into Fibrin Gels

The capacity of the chondrocytes to migrate into a fibrin gel was investigated as described previously [27]. Briefly, a piece of cartilage was placed in a 24-well, non-tissue culture plate in a 4-mm-diameter polydimethylsiloxane mold that was subsequently filled with 25 µL of fibrin glue (2 mM CaCl2, 1 U/mL thrombin, 20 mg/ml fibrinogen. After crosslinking for 20 minutes in the incubator (37° C, 5% CO_2, 95% humidity), 1 mL of culture medium was added onto the scaffolds, which were cultured in free-floating conditions for 4 weeks and subsequently fixed in 4% paraformaldehyde for 1 hour. After cell permeabilization with Triton X-100, the gels were stained with 4', 6-diamidino-2-phenylindole (DAPI) and phalloidin-rhodamine for 1 hour in the dark at room temperature. Whole gels were imaged with a Zeiss Axio Explorer using the ApoTome.2 (Carl Zeiss Microscopy, Munich, Germany) at 10 x magnification. The projected area of the gel covered with outgrown cells was quantified manually with Fiji software, and the migration area was calculated as the area

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Kommentiert [TjG4]: AU: Did you mean INTERobserver reliability here? Did you perform a kappa/ICC analysis? ED

AU response: thank you for noticing the word error, we indeed meant "interobserver". No kappa/ICC analysis was perfomed since the observations were entirely descriptive and did not rely on a scoring system.

hat gelöscht: ra

Kommentiert [CO5]: AU: Please parenthetically provide the manufacturer name and location (city, state, and country).

Author response: this information was added

covered with outgrown cells divided by the total area available to the cells to outgrow (total area of the gel minus the area of the cartilage piece). Although the contrast of the cells on background is clear enough to delineate the migration area, the manual aspect of this quantification can lead to slight variability in the results. However, the goal of this measurement is to give an idea of the migration capacity of these cells within the pool of replicates and does not provide an exact threshold for a clinical recommendation.

Semi-quantitative and Qualitative Assessment of Flap Chondrocytes Ability to Produce Cartilaginous Matrix - Chondrocyte Isolation, Expansion, and Differentiation in Pellets

The chondrocytes pellet assay is a 3-D culture system, originally developed to test the chondrogenic potential of mesenchymal progenitor cells [22]. The assay mimics cell condensation during limb development and is widely used to test the capacity of chondrocytes to deposit cartilaginous matrix in a 3-D environment. Chondrocytes were isolated by digesting minced cartilage samples with collagenase overnight [14]. Chondrocyte viability in the resulting cell suspension was determined using a standard trypan blue exclusion assay, before plating the cells in a T25 flask and passaging them once at 80% confluency. Cells were then trypsinized once more (cells in pellets were thus at Passage 2) and 250,000 cells were pipetted and spun in conical bottom 96-well plates to form pellets, subsequently cultured for 4 weeks in chondrogenic medium containing 10 ng/mL transforming growth factor β3. Of note, control samples were healthy knee chondrocytes isolated from samples of patients of the same age range. Although these cells originated from a different joint, they have been shown in a previous study to produce cartilage in pellet culture at passage 2 [14], thus constituting good positive controls. Lastly, pellets were embedded in an optimal cutting temperature compound, snap frozen and cut with a cryostat into 5-µm-thick sections before staining as described above in the histology section.

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196 *Statistical Analysis*

197 All experiments were performed with material from a minimum of five patients with FAI
198 (due to limited amount of tissue, a sample could most often not be used for all four assays),
199 with at least two (outgrowth) and three (viability) technical replicates for quantitative assays.
200 Due to the exploratory nature of this study, the effect size could not be estimated, a
201 requirement for sample size calculation. Considering the limited availability of chondral
202 flaps, the study was performed on a relatively small cohort of patients. For viability
203 quantification, histology, and pellet assays, three controls were used due to the restricted
204 availability of healthy-looking tissue. The results of the quantitative assays are reported as the
205 mean ± SD of the biological replicates (each single point is the average of the technical
206 replicates of each patient's sample). Statistical tests were performed with Prism (GraphPad
207 Software Inc, version 7.0.3). Normality was verified with the Shapiro-Wilk test. Because
208 sample sizes were unequal, Student t-tests with Welch's correction were used to compare two
209 groups. To compare the results of the two viability assays (live-dead versus trypan blue), a
210 paired, two-tailed t-test was performed. The correlation between viability and age was
211 evaluated with a non-parametric Pearson rank correlation test. Statistical significance was set
212 at $p < 0.05$.

213 **Results**

214 *Cartilage Flaps Contain Fewer Viable Chondrocytes than Non-delaminated Hip Cartilage*
215 *Samples*

216 In delaminated flaps, chondrocyte viability was decreased when compared with non-
217 delaminated hip cartilage samples (50% ± 19% versus 76 ± 6%; means difference -26 [95%
218 CI -46 to -6]; p = 0.02) (Fig. 3). There was no correlation between viability and the age of the
219 patients (r = -0.19; p = 0.7). Viability quantification with a trypan blue exclusion assay on a

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suspension of freshly isolated chondrocytes led to higher values than with live-dead staining (84% ± 9% versus 54% ± 18% in the same five chondral samples; mean difference 30 [95% CI 4 to 56]; p = 0.03) (Supplemental Fig. 1; supplemental materials are available with the online version of *CORR*®).

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Chondrocytes in Chondral Flaps Reside in a Disrupted ECM

In all seven flap samples tested for histology, the qualitative assessment of the tissue organization with H&E staining showed the presence of a fibrillated ECM, which was present either throughout the sample or located at the surface of the cartilage towards the delaminated edge of the flap, suggesting a gradient of degeneration (Fig. 4). Fibrillation was associated with a loss of normal tissue stratification; that is, neither flattened cells in the superficial layer nor columnar cells in the deeper layers were visible. In addition, this fibrillated area correlated with a loss of GAG (no Safranin O staining). The gradient of GAG in healthy cartilage, characterized by a progressively increasing amount of GAG toward deeper layers, was consistently lost in all flat samples. Some GAG was retained along the attached side in some samples, whereas in some samples, GAG could not be detected (Fig. 4). The loss of GAG was not accompanied by a loss of collagen, as suggested by the strong intensity of the picrosirius red staining, an anionic dye that associates to the cationic collagen fibrils (data not shown). Patches of Type I collagen-positive staining were visible in two of the seven flap samples used for histology, but only in fibrillated areas (Fig. 4). All samples had positive staining results for Type II collagen, which was mostly present in the superficial zone and detected only pericellularly in the middle and deep zones of healthy cartilage. This localization was lost in the flap samples, where Type II collagen was present throughout the thickness, with a higher staining intensity than in healthy controls.

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Chondrocytes Are Able to Migrate Out of Chondral Flaps into Fibrin Gels

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Chondrocyte migration out of the chondral ECM was evaluated semi-quantitatively upon encapsulation in fibrin gels. The presence of cells migrating and spreading within the fibrin gel was observed in all five flap samples tested. The capacity of the cells to "colonize" the fibrin gel was further quantitatively assessed by measuring the proportion of the projected area of the gel covered with migrating cells. This quantification showed that the mean proportion of the gel where migrating chondrocytes were present was 71 ± 25%. In other terms, the fibrin gel supported the outgrowth and the proliferation of flap chondrocytes, resulting in an extended coverage of the gel with cells (Fig. 5A-B). These observations indicate that the ECM permitted chondrocyte migration into fibrin glue, possibly because of the above-described degraded state of the ECM [30]. There was variability among patients and among the technical triplicates for each patient's sample (two or three independent fibrin gels were made for each flap sample) (Fig. 5C).

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Cells Isolated from Flaps have a Lower Capacity to Deposit Cartilaginous Matrix than Controls

To semi-quantitatively describe the capacity of flap chondrocytes to produce cartilaginous matrix, which would be important for the long-term fixation of the flap (considering the low stability of fibrin sealants in vivo) and for the flap tissue repair once refixated, we compared pellets of chondrocytes isolated from either flaps or from controls. Of the five biological replicates for pellets isolated from flaps, four led to the formation of pellets with ECM production, while one showed almost no matrix production (Fig. 6A). The qualitative assessment of cell distribution show that cells were less homogeneous in pellets isolated from flaps (presence of "cell patches") compared with control samples, as shown by the hematoxylin counterstain. We also observed that the ECM produced in pellets from controls was homogeneously distributed (Fig. 6B), in contrast to pellets from flaps, which displayed

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268 "holes" of fainter staining. Type I collagen was present in all pellets. In addition, the pellets in
269 flaps were smaller than those in controls (Fig. 6D), as shown by their lower average surface
270 area compared to pellets from control samples (0.4 mm^2 ± 0.09 versus 0.7 ± 0.1 mm^2
271 respectively, mean difference 0.3 [95% CI, 0.03 to 0.6]; p = 0.038). This observation,
272 together with the inhomogeneous patches of cells, suggests that flap chondrocytes
273 proliferated less compared to "healthy" knee chondrocytes, previously found to have a good
274 chondrogenic potential.

275 **Discussion**

276 Recent clinical and experimental work has suggested that cam-type FAI frequently causes
277 cartilage delamination and the subsequent development of osteoarthritis of the hip. To avoid
278 such cartilage damage in severe cases of cam FAI, surgery may be advised [1]. During such
279 procedures, delamination of the acetabular cartilage is often encountered and the damaged
280 cartilage is generally resected. Based on the reported presence of various amounts of viable
281 cells in chondral flaps [11, 18, 35, 43], refixation of these flaps with a fibrin sealant has also
282 been suggested as an alternative to resection. However, there is no well-defined scientific
283 rationale supporting this technique. Legitimate questions regarding the tissue characteristics
284 remain, notably whether there are enough viable cells in the flaps, whether the structure, and
285 therefore the function, of the flap tissue is preserved, or whether the residing cells are able to
286 provide solid attachment to the subchondral bone. The present study augments cell viability
287 and histology data with a study of chondrocytes' capacity to outgrow from the ECM into
288 fibrin gels and produce a cartilaginous ECM. Our main findings are (1) a lower percentage of
289 viable chondrocytes were present in flap cartilage compared with controls; (2) the ECM in
290 flaps displayed an altered structure at the delaminated edge of the flap or throughout the
291 whole flap in some cases, probably induced by repeated mechanical insult; (3) the

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chondrocytes could migrate out of the flap tissue into fibrin glue; and (4) flap chondrocytes have a limited capacity to produce ECM. These findings challenge the refixation practice and suggest the need for further mechanistic large animals and clinical studies focusing on the long-term outcome of flap refixation based on follow-up MRI.

Limitations

We are aware that our study has several limitations. First, although the sample size is similar to previous studies investigating chondral flaps [11, 18, 35], it remains too small to generalize the results. This in vitro study is meant to be exploratory and to stimulate re-evaluation of this clinical practice, rather than to provide a definitive surgical recommendation. Large animal studies would provide insights into the repair mechanism and the long-term efficiency. Additionally, the samples were small, which limited the number of assays that could be done per sample. For instance, the cell yield after chondrocyte isolation and expansion in vitro was up to 720,000 cells at Passage 2, which illustrates the small amount of living cells initially present in the flaps and the need for a large amount of tissue to perform pellet assays (250,000 cells per pellet). Despite this limitation, the sample size for each experiment was at least 5 biological replicates, and the consistent results between these replicates can give confidence into the presented results.

Another limitation is the use of two different types of controls. For the viability and ECM composition assays, the goal was to assess whether the flap displayed a lower quality than non-delaminated cartilage. Three non-delaminated acetabular cartilage samples could be obtained. This low number of controls as compared with flaps is directly linked to the limited availability of healthy-looking cartilage in these patients, and does not represent an obstacle to performing statistical tests. These samples originated from hips of patients undergoing surgery (arthrodesis, derotational femoral osteotomy and THA) with acetabular cartilage

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appearing well fixed while the femoral head cartilage was affected (AVN of femoral head and anterior and lateral hip instability with suprafoveolar head damage). Histological staining of these controls displayed all the known characteristics of healthy acetabular cartilage at the tissue level and we, therefore, considered these as a fair control tissue. The question of flap chondrocytes' ability to deposit ECM was a yes/no question. Therefore, we included a positive control, namely knee chondrocytes known to effectively deposit matrix, to describe the features of pellets made of cells displaying a good chondrogenic potential. In a previous study, our laboratory has shown that the knee chondrocytes from donors in the same age-range as the FAI patients included in this study, could deposit a good quality of cartilaginous matrix at Passage 2 [14]. Consequently, although the mechanical environment of knee and hip cartilage is different, these knee chondrocytes are acceptable as a positive control in terms of chondrogenesis. It is important to mention that the pellet assay cannot fully predict the behavior of cells that migrated in the fibrin gel, given the 2-D in vitro expansion step required to obtain enough cells. Flap chondrocytes were shown to dedifferentiate during in vitro culture but to re-differentiate upon a switch to 3-D culture [11]. The pellet assay, although not using a hydrogel as a matrix, still provides a 3-D culture environment, which may compensate for the need of expansion. The observations in pellets can, however, be strengthened by the finding that the gels used for the migration assay were very soft and difficult to handle after the 4 weeks of culture, which strongly suggests the absence of matrix produced by the migrating cells. It is evident that the free-floating condition during the culture of the fibrin gels drastically differs from a hip environment [20, 26]. The free-floating condition can be a reason for absence of deposited ECM, but it is necessary to investigate specifically the property of the fibrin sealant to support migration of the cells growing out of the flaps.

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340 It was not possible to exactly define the duration of the mechanical degradation, but all
341 patients were symptomatic for at least 1 year (Table 2). Considering the fact that the onset of
342 FAI symptoms appear once advanced structural changes have happened, we assume that
343 patients had lived at least 1 year with damaged articular cartilage. All patients had cam FAI
344 (Fig. 2), which minimized population heterogeneity.

345 *Chondrocyte Viability in Cartilage Flaps and Quality of ECM*

346 We found lower chondrocyte viability in delaminated flaps compared with control tissues
347 (50% ± 19% versus 76 ± 6%), confirming other reports of low viability in flaps (39% ± 14%
348 [18] and 54.6% ± 25.6% [35]), although these data contrast with the high viability (87% ±
349 10%) found by Wright et al. [43]. We found there was higher viability in non-delaminated
350 controls, which was not shown in an earlier report (38.8%) [35]. The presence of viable cells
351 is important to consider when deciding whether to re-fixate chondral flaps since residing
352 chondrocytes are key players in the regeneration process, through migration and proliferation
353 [28, 34]. The variable results found in previous studies may be attributed to the quantification
354 technique, because we observed higher viability with the trypan blue exclusion assay on
355 isolated cells (Supplemental Fig. 1; supplemental materials are available with the online
356 version of *CORR*) than with the live-dead assay on whole cartilage samples. Finally, if the
357 reported viability in allografts after the maximum allowed storage time of 45 days (~ 66%
358 [39]) was considered as a threshold for tissue implantation or refixation, the viability in flaps
359 would be too low to be considered for refixation.

360 Our study showed that chondrocytes in chondral flaps exist in a degraded ECM environment.
361 A novel aspect of this work is the observation of the flaps in their entirety, which highlighted
362 a loss of GAG and the presence of fibrillation either only at the delaminated edge of the flap
363 or in the whole sample in the worst cases. The structural organization of the ECM, crucial for

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the function of the cartilage as a load absorber [16], is lost throughout the flap, not only at the surface or delaminated edge. These features likely revealed a mechanically damaged tissue, as described by Kurz et al. [24]. The presence of various amounts of degenerated, fibrillated tissue in chondral flaps has already been reported, although without consideration of the whole flap structure [23, 35]. Our study confirmed the GAG depletion [18, 35], loss of columnar distribution of the cells, and the disorganized structure of the ECM [20, 35] that has been described. The drastic loss of GAG provides an environment in which cells are more prone to die in case of mechanical injury [32], which could explain the low viability observed. Our findings regarding the increased area stained with Type II collagen in all tested samples are in line with a published biochemical quantification of collagens in flaps, which showed increased amounts of collagen in flaps that have a degenerated gross appearance [18]. However, previously published histology data in flaps showed a loss of Type II collagen in flap samples with high Osteoarthritis Research Society International grades [35]. Increased Type II collagen production seems more consistent with the known correlation between mechanical stimulation and anabolic metabolism of chondrocytes [8] on the one hand, and between FAI and development of osteoarthritis on the other. During the early stage of osteoarthritis, the collagen synthesis rate increases [41] and a transition from Type II to Type I collagen occurs [25], which motivated our assessment of the presence of Type I collagen, as it had not been done before. We observed the presence of Type I collagen in two of seven samples in the form of islands; one of these two samples originated from a patient with a Tönnis Grade of 1.

Regeneration Potential of Cells in Cartilage Flaps, in Terms of Migration and Matrix Deposition

We observed chondrocyte migration out of the flap ECM into fibrin in all five biological

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samples, notwithstanding the relatively high fibrin concentration in gels, which is used to increase the gel's stability [15]. Many in vitro studies have investigated the capacity of chondrocytes to migrate from healthy tissue into fibrin glue as a repair strategy for cartilage lesions [3, 9, 42, 44]. Absence of outgrowth has been reported [3, 44], unless additional mechanical [42] or chemical [9] cues were provided. The reported need for an ECM digestion step to promote cellular outgrowth from a healthy ECM in cartilage [29, 33, 36] strengthens our theory that the loss of GAG and degenerative features in flap samples promote outgrowth. This could be an argument in favor of refixation; however, the fibrin gels after culture were very soft, which is most probably because of the absence of ECM production by the outgrown cells. We thus investigated the capacity of residing chondrocytes to produce cartilaginous matrix with a pellet assay.

When testing the ECM deposition by cells isolated from flaps, we observed not only limited deposition of cartilaginous matrix but also Type I collagen. Pellets of flap chondrocytes displayed smaller sizes as well as less-homogeneously distributed cells and ECM compared with positive control pellets. The limited capacity to produce cartilaginous ECM, even in the presence of supraphysiologic amounts of chondrogenic transforming growth factor β3, is a drawback to the refixation of flaps as they may not be able to deposit ECM within the fibrin sealant to strengthen the fixation to the subchondral bone. These findings are in contrast to those of Giannini et al. [17] who demonstrated the promising use of cells harvested from detached osteochondral fragments for autologous chondrocyte implantation procedures in the ankle. However, in their study, the histologic appearance of the detached tissue appeared to be hyaline-like, in contrast to the disrupted ECM of the flaps. Biant and Bentley [7] investigated the chondrogenic potential of cells isolated from cartilage debrided during autologous chondrocyte implantation and cultured until Passage 2. They showed that DNA,

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GAG, and Type II collagen amounts in pellets of chondrocytes from debrided samples were similar to the amounts obtained in pellets of chondrocytes from healthy, non-load-bearing cartilage samples. The results of Bretschneider et al. [11] also indicate that flap chondrocytes de-differentiate but can recover their phenotype upon encapsulation in a chondro-inductive biomaterial. Nevertheless, fibrin was shown to fail at promoting chondrocyte redifferentiation [6], which challenges the use of this biomaterial. Of note, the study of ECM deposition by flaps chondrocytes cannot predict whether the flap will heal upon refixation, due to the technical limitation of chondrocyte expansion in vitro. However, it can be expected that the flaps would not heal, considering the well-known limited capacity of cartilage tissue to regenerate upon damage [12] and the very slow collagen turnover in healthy and pathologic joints [19]. Studies reporting results of flap refixation do not focus on the quality of the refixated tissue or the newly generated tissue at the interface with the subchondral bone, but rather on clinical scores [37, 40].

Conclusions

We found that chondrocytes isolated from hip flaps can outgrow into fibrin glue, however, their cell viability is low (< 70%) and they have limited capacity to deposit cartilage matrix. These data suggest that the fibrin refixation approach should be questioned, as it may not lead to long-term attachment and repair; however, the results should be considered cautiously due to the small sample size. A study in a large animal model would give more realistic insights into the long-term efficacy of refixation with fibrin to ultimately provide a more definitive surgical recommendation.

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Acknowledgments

We thank Prof. Dr. med Matthias Steinwachs for providing the knee cartilage samples and Dr. Emma Cavalli for isolating the chondrocytes from these knee cartilage samples. The authors acknowledge the use of the Scientific Center for Optical and Electron Microscopy for histology imaging. Finally, we thank Angela Bonato, MSc and Benjamin Kessel, MSc for their blinded observations of the histological sections.

Kommentiert [CO11]: AU: If possible, please provide the final academic degree for each of these individuals.

Author response: The two missing titles were added.

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Legends

Fig. 1 This figure shows an overview of the study's procedures. (A) Chondral flaps were harvested from the hips of adult patients with FAI and sutured on the delaminated edge. (B) We used oriented hip flaps to investigate the properties of (C) whole tissue pieces (viability and ECM distribution), (D) particulated pieces embedded in fibrin glue, casted in a PDMS (polydimethylsiloxane) for the migration assay, and (E) isolated chondrocytes cultured in vitro (chondrogenesis in pellets).

Fig. 2 Radiographs of hips from patients included in the study. The sex (M/F) and age are indicated at the top of the radiograph. The sample number is placed at the bottom right of the images.

Fig. 3 Chondrocyte viability was decreased in flaps samples compared with controls. (A) These representative images show the (I) highest and (II) lowest microscopic images of live-dead staining of non-delaminated hip samples (top) and flaps (bottom). Live cells are depicted in green and dead cells in red. The sex, age, and viability result are given at the bottom left. Scale bar: 100 µm. (B) This summary table shows the viability results for each sample (six biologic replicates for flaps and three for controls, with three technical replicates), obtained from quantification of live-dead images. (C) This plot shows the viability results (dots represent the average of the three technical replicates of each donor analyzed), comparing viability between non-delaminated controls and flaps (unpaired t-test with Welch's correction, * $p < 0.05$).

Fig. 4 The ECM structure was fibrillated and depleted of glycosaminoglycan (GAG) in chondral flaps. Histologic staining of 5-µm-thick sections of oriented flap samples are shown. From left to right: these images show hematoxylin and eosin, Safranin O (GAG stains), and immunohistochemistry results for Type II and Type I collagen (hematoxylin counterstain,

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Kommentiert [CO12]: AU: In the figure, please use sentence case for all text, meaning that only the first letter of the first word is capitalized, unless there is a proper now.

In Fig. 1C, please delete the ampersand symbol and use the word "and" instead.

Author response: We implemented the changes, notably by capitalizing the following:
- Fig. 1A: "Marks"
- Fig. 1C: "Superficial"; "Attached"
- Fig. 1D: "Minced" and "Casting"
- Fig. 1E: "Isolation", "Expansion" and "Centrifugation"

Kommentiert [CO13]: AU: In the figure, please capitalize the first letter of "Female" and "Male" at each instance.

Please put a space before and after the equal sign in the p value.

Please include a note about the asterisk in the legend.

Author response: we capitalized the letters as requested and added the spaces around the "=" sign. The note about the asterisk was added in the legend in replacement of the exact p value, which is already mentioned in the figure.

hat gelöscht: p = 0.02,

positive staining in brown). The * is positioned at the side of delamination, on the side of the acetabular cartilage surface. The first row shows the staining of a non-delaminated hip cartilage sample and the three following rows show three representative images of flap samples (sex and age indicated on the left side), from the best- (M21) to the worst- (M32) looking conditions, from top to bottom. On the hematoxylin and eosin staining pictures, the dotted line separates the fibrillated and smooth areas. n = 7 oriented samples and n = 3 control samples. Scale bars: 500 µm; H&E = hematoxylin and eosin.

Fig. 5 This figure shows the outgrowth potential of chondrocytes in flap samples. (A) A scoring system was used to investigate the outgrowth potential of the cells. Gels were imaged with 10 x magnification, and outgrowth values were calculated as the ratio of the area covered with cells (Ac) to the total area of the gel (Ag). (B) This plot shows the outgrowth score (five biologic replicates with two or three technical replicates for each biologic replicate). (C) These image shows an example of the best score (left), an intermediate score (middle), and a low score (right). Scale bars: 500 µm.

Fig. 6 Chondrocytes from chondral flaps display low chondrogenic potential. These images show histologic staining of 5-µm-thick cryosections of pellets after a 4-week culture period in vitro for glycosaminoglycan (GAG) (Alcian blue) and Types I and II collagen. (A) This image shows pellets made of flap chondrocytes at Passage 2, n = 5. (B) This shows pellets made of chondrocytes from non-delaminated knee articular cartilage samples—Knee AC at Passage 2, n = 3. Two negative controls (no primary antibody) are shown for immunostaining (bottom right). Sex and age (years) are indicated at the bottom left of all images. Arrows indicate areas where fainter or absent staining was observed, and dotted lines circle cell clusters. Scale bar: 100 µm (close up) and 500 µm (inset). (C) This image shows the immunohistochemistry of the negative control sample (no primary antibody). Scale bar: 100

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Kommentiert [CO14]: AU: Please put a space before and after the equal sign at each instance.

Author response: we added the spaces

Kommentiert [CO15]: AU: Please put a space before and after the equal sign in the p value.

Author response: this was done

µm (close up) and 500 µm (inset). (D) This plot shows the measured area of the pellets, determined with an unpaired t-test with Welch's correction.

Supplemental Fig. 1 The chondrocytes' viability depends on the quantification technique. (A) This plot shows the viability results (dots are the average of the three technical replicates of each donor analyzed), comparing viability of the flaps using either live-dead or trypan blue exclusion assays (paired t test, * $p < 0.05$). (B) This is a summary table of viability results for each sample (five biological replicates, three technical replicates for each).

Kommentiert [CO16]: AU: In the actual image, please use sentence case for capitalizing words (that is, only capitalize the first letter of each word.

Please put a space before and after the equal sign in the p value.

Please explain the asterisk in the A image in the legend.

For the column labeled "Age", please spell out "years" and put it in parentheses.

Please change "Gender" to "Sex", and include (M/F) in the column heading.

Please provide measurement labels for the columns "Viability live-dead" (note the addition of the hyphen; that is how you have written it in all other instances) and "Viability trypan blue"; are the measurements percentages? If so, include this in the column head: (%). It helps the reader to immediately understand the data you are presenting.

Author response: thank you for all the suggestions, all modifications were made

hat gelöscht: p = 0.03,

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