

# Novel ex vivo human osteochondral explant model of knee and spine osteoarthritis enables assessment of inflammatory and drug treatment responses

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**Novel Ex Vivo Human Osteochondral  
Explant Model of Knee and Spine  
Osteoarthritis Enables Assessment  
of Inflammatory and Drug Treatment  
Responses**

GRADUATE THESIS



Zagreb, 2019.

This graduate thesis was realized at two locations. At the division of orthopaedics; Department of Orthopaedic Surgery and Traumatology, Clinical Hospital "Rebro", School of Medicine, University of Zagreb, under the supervision of Mislav Jelić, MD, PhD. It was also realized at the Biomolecular Research Laboratory of the Department of Spine Surgery at the University Hospital Basel under the supervision of Jeroen Geurts, PhD. The project was carried out at the interdisciplinary Department of Biomedical Engineering and involved collaboration with spine surgeons, bone biologists and radiological imaging experts. The paper was published in the Journal of Molecular Sciences (<https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5983625/> "Novel Ex Vivo Human Osteochondral Explant Model of Knee and Spine Osteoarthritis Enables Assessment of Inflammatory and Drug Treatment Responses," by J. Geurts, D. Juric, and M. Muller, International Journal of Molecular Sciences. 2018 Apr 28;19(5). pii: E1314. doi: 10.3390/ijms19051314.) on 28<sup>th</sup> of April and was submitted as a thesis paper for evaluation in the academic year of 2018/2019.

Mentors of the graduate thesis; Mislav Jelić, MD, PhD and Jeroen Geurts, PhD

## **Abbreviations**

DMOAD: Disease-modifying osteoarthritis drug

OA: Osteoarthritis

LPS: Lipopolysaccharide

TGF- $\beta$ : Transforming growth factor- $\beta$

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## 1. Summary

### NOVEL EX VIVO HUMAN OSTEOCHONDRAL EXPLANT MODEL OF KNEE AND SPINE OSTEOARTHRITIS ENABLES ASSESSMENT OF INFLAMMATORY AND DRUG TREATMENT RESPONSES

Doria Jurić

**Abstract:** Osteoarthritis of the knee and spine is highly prevalent in the modern society, yet a disease-modifying pharmacological treatment remains an unmet clinical need. A major challenge for drug development includes selection of appropriate preclinical models that accurately reflect clinical phenotypes of human disease. The aim of this study was to establish an ex vivo explant model of human knee and spine osteoarthritis that enables assessment of osteochondral tissue responses to inflammation and drug treatment. Equal-sized osteochondral fragments from knee and facet joints (both n = 6) were subjected to explant culture for 7 days in the presence of a toll-like receptor 4 (TLR4) agonist and an inhibitor of transforming growth factor-beta (TGF- $\beta$ ) receptor type I signaling. Markers of inflammation, interleukin-6 (IL-6) and monocyte chemoattractant protein-1 (MCP-1), but not bone metabolism (pro-collagen-I) were significantly increased by treatment with TLR4 agonist. Targeting of TGF- $\beta$  signaling resulted in a strong reduction of pro-collagen-I and significantly decreased IL-6 levels. MCP-1 secretion was increased, revealing a regulatory feedback mechanism between TGF- $\beta$  and MCP-1 in joint tissues. These findings demonstrate proof-of-concept and feasibility of explant culture of human osteochondral specimens as a preclinical disease model, which might aid in definition and validation of disease-modifying drug targets.

**Keywords:** Osteoarthritis; osteochondral; experimental model; inflammation; bone metabolism; knee; spine

## 2. Sažetak

### NOVI EX VIVO HUMANI OSTEOHONDRALNI EKSPLANTACIJSKI MODEL KOLJENA I KRALJEŽNICE OMOGUĆUJU PROCJENU UPALNOG I TERAPIJSKOG ODGOVORA

Doria Jurić

**Sažetak:** Osteoartritis koljena i kralježnice vrlo je čest u modernom društvu, no farmakološko liječenje koje utječe na bolest ostaje i dalje neispunjena klinička potreba. Glavni izazov za razvoj lijeka uključuje odabir odgovarajućih predkliničkih modela koji točno odražavaju kliničke fenotipove bolesti. Cilj ovog istraživanja bilo je utvrditi ex vivo model eksplantacije osteoartritisa ljudskog koljena i kralježnice koji omogućuju procjenu odgovora osteokondralnog tkiva na upalu i liječenje lijekovima. Osteokondralni fragmenti jednakih veličina iz zglobova koljena i faceta (oba n=6) podvrgnuti su kulturi eksplantacije 7 dana u prisutnosti agonista TLR4 i inhibitora TGF- $\beta$  receptora tipa I. Markeri upale, interleukin 6 (IL-6), monocytno-atraktantni protein-1, ali ne i metabolizam kosti (pro-kolagen-1) bili su značajno povišeni liječenjem s TLR4 agonistom. Ciljanje TGF- $\beta$  signalizacije rezultiralo je jakom redukcijom pro-kolagena-1 i značajno smanjilo razine IL-6. MCP-1 sekrecija je povećana, otkrivajući regulatorni mehanizam povratne sprege između TGF- $\beta$  i MCP-1 u zglobovnom tkivu. Ovi nalazi dokazuju, koncept i izvedivost kulture eksplantacije ljudskih osteokondralnih uzoraka kao predklinički model bolesti, koji bi mogao pomoći u definiranju i validaciji ciljeva lijekova koji modificiraju bolesti.

**Ključne riječi:** Osteoartritis; osteokondralni; eksperimentalni model; upala; metabolizam kosti; koljeno; kralježnica

### 3. Introduction

Osteoarthritis (OA) is a chronic disorder involving movable joints that is characterized by cell stress and extracellular matrix degradation initiated by micro- and macro-injury that activates maladaptive repair responses including pro-inflammatory pathways of innate immunity [1]. The prevalence of radiographic OA is high in facet joints of the lumbar spine and the knee joints of elderly individuals and is associated with age and obesity [2–4]. While the etiopathogenesis of OA still remains unknown, it has been established that pathological changes to several tissues including articular cartilage, synovium and subchondral bone and marrow are involved in joint degeneration [5–7]. The presence of synovial inflammation and bone marrow lesions is strongly associated with the progression of knee and facet joint osteoarthritis in humans [8–10]. OA severity is correlated with increased expression of a number of pro-inflammatory mediators, including interleukin-6 (IL-6) and monocyte chemoattractant protein-1 (MCP-1/CCL2) [11,12]. It has been shown in experimental and human OA that agonists of Toll-like receptor 4 (TLR4), such as lipopolysaccharide (LPS) and damage-associated molecular patterns (DAMPs) produced in the degenerated joint, play a central role in the inflammatory response in diseased joints [13–16]. Nevertheless, a disease-modifying OA drug (DMOAD) is still lacking, and total joint replacement remains the standard symptomatic treatment for end-stage disease.

Definition of novel drug targets and preclinical evaluation of DMOADs predominantly relies on the use of *in vitro* models, including isolated chondrocytes and osteoblasts from human OA tissues, or experimental murine models [17,18]. Animal models have demonstrated promising results for therapeutic treatment of knee OA based on inhibition of transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1) in subchondral bone, and Adamts5 or WNT/ $\beta$ -catenin signaling in articular cartilage [19–21]. However, these findings have not yet resulted in a DMOAD therapy for OA in humans. Several limitations of *in vitro* and experimental models pose serious challenges to the translation of preclinical findings into clinical practice. While different clinical phenotypes are known in humans, including inflammatory, metabolic and biomechanical OA [22], experimental models are predominantly surgically induced post-traumatic OA [23]. The vast majority of *in vivo* models are focused on knee OA, and validated models for spine, hand and hip OA are lacking.



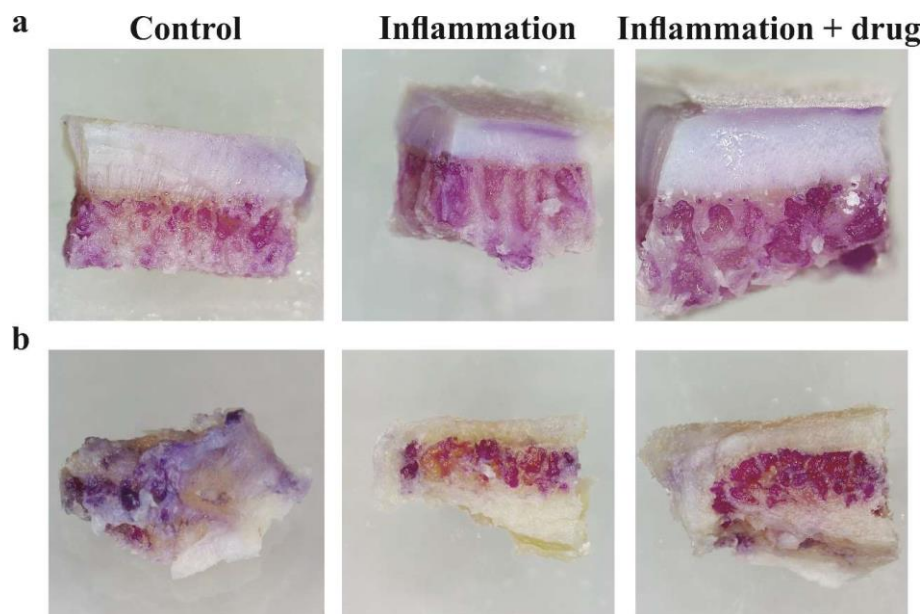
In addition, the relatively small size of murine joints complicates the assessment of bone marrow lesions and synovitis by magnetic resonance imaging, which are important diagnostic and prognostic imaging biomarkers in human OA [6]. Experimental in vitro studies with isolated chondrocytes and osteoblast provide valuable insight into cellular responses, but do not take the crosstalk between different joint tissues into account. Tissue culture models of isolated articular cartilage from OA specimens have proven valuable in functional studies and detection of pathological hallmarks [17,18]. There is a paucity of models that focus on additional joint compartments, including subchondral bone and marrow tissue. The challenges for future DMOAD development include recognition of OA as a complex disease with multiple phenotypes and potential joint-specific pathomechanisms [24].

The aim of our study was to establish an ex vivo osteochondral tissue culture model of human knee and facet joint OA that is responsive to an inflammatory challenge and enables the assessment tissue responses to drug treatment effects. Treatment with a TLR4 agonist led to upregulated secretion of IL-6 and MCP-1 proteins, while leaving bone metabolism, assessed by pro-collagen-I (pro-Col-I), unaffected. Inhibition of TGF- $\beta$  receptor type I signaling significantly reduced pro-Col-I and IL-6 secretion in knee and facet joint specimens but led to increased MCP-1 levels. These findings provide proof-of-concept and feasibility of explanted osteochondral clinical specimens as preclinical human and joint-specific OA model.

## 4. Results

### 4.1. Tissue Viability after Explant Culture

Osteochondral specimens were prepared from osteoarthritic knee tibial plateaus or facet joints. Cancellous bone from iliac crest and distal lateral tibial plateau served as osteal tissue controls. Samples were cultured in osteogenic culture medium with and without an inflammatory stimulus and drug treatment, and tissue viability was evaluated by assessment of cell metabolic activity using MTT staining (Figure 1). Viable cells were readily detected in subchondral bone marrow and cartilage tissues. Gross evaluation of staining patterns and intensity revealed no reduced tissue viability under inflammatory or drug treatment conditions.



**Figure 1.** Tissue viability after explant culture of osteochondral specimens from (a) knee and (b) facet joint osteoarthritis. Fresh clinical specimens were cut in equal-sized fragments and cultured in osteogenic culture medium for one week. Samples were either left untreated (control) or challenged with 1  $\mu\text{g}/\text{mL}$  lipopolysaccharide (inflammation) in the absence of a drug treatment (10  $\mu\text{M}$  TGF- $\beta$  receptor type I inhibitor). Adapted [reprinted] from “Novel Ex Vivo Human Osteochondral Explant Model of Knee and Spine Osteoarthritis Enables Assessment of Inflammatory and Drug Treatment Responses,” by J. Geurts, D. Juric, and M. Muller, *International Journal of Molecular Sciences*. 2018 Apr 28;19(5). pii: E1314. doi: 10.3390/ijms19051314.

#### **4.2. Secretion of Pro-Collagen-1 and Inflammatory Mediators under Basal and Inflamed Conditions**

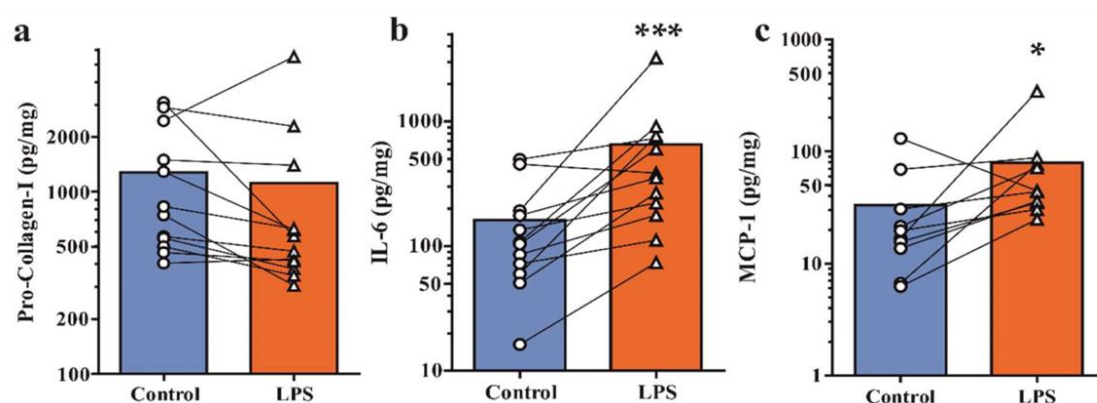
Protein levels of pro-collagen-1 (pro-Col-1), as marker of bone metabolism, and inflammatory cytokine (IL-6) and chemokine (MCP-1) were determined in tissue-conditioned medium by ELISA. All specimens secreted pro-Col-1, IL-6 and MCP-1 under basal conditions. Weight-normalized expression levels of non-osteoarthritic cancellous bone were approximately tenfold higher than osteoarthritic osteochondral specimens. Next, we assessed whether explanted tissue specimens were responsive to an inflammatory insult. Samples were challenged with Toll-like receptor 4 agonist lipopolysaccharide (LPS, 1 microgram per mL), which has been found in synovial fluid inflamed OA knee joints and mimics signalling induced by damage-associated molecular patterns that are present in degenerative joints. Pro-Col-1 expression were unaffected by LPS challenge in osteoarthritic specimens. Protein levels of the inflammatory mediators IL-6 and MCP-1 were 4-fold and 2.4-fold upregulated in knee and facet joints. A similar response was observed in non-osteoarthritic controls, which showed unaffected pro-Col-1 levels and 1.5- and 2.4-fold upregulation of IL-6 and MCP-1 respectively. These findings demonstrate that osteochondral tissue specimens are capable of mounting an inflammatory response under ex vivo culture conditions.

**Table 1.** Weight-normalized basal secreted protein levels of non-osteoarthritic controls and osteoarthritic knee and facet joint specimens.

Secreted Protein	Total OA ( <i>n</i> = 12)	Facet OA ( <i>n</i> = 6)	Knee OA ( <i>n</i> = 6)	Osteal Tissue ( <i>n</i> = 5)
pro-Col-I (pg/mg)	1273 ± 287	1660 ± 522	886 ± 172	7392 ± 3604 <sup>†</sup>
IL-6 (pg/mg)	163 ± 45	223 ± 83	102 ± 22	1970 ± 1368 <sup>†</sup>
MCP-1 (pg/mg)	33 ± 13	49 ± 23	17 ± 4	437 ± 287 <sup>†</sup>

<sup>†</sup> *p* < 0.05 compared with OA osteochondral specimens by ANOVA; OA, Osteoarthritis.

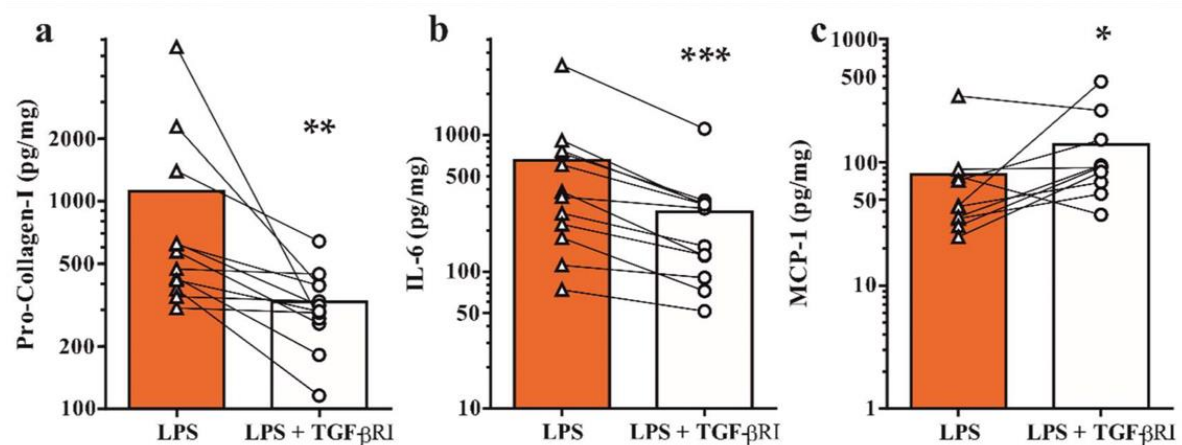
*Note.* Adapted [reprinted] from “Novel Ex Vivo Human Osteochondral Explant Model of Knee and Spine Osteoarthritis Enables Assessment of Inflammatory and Drug Treatment Responses,” by J. Geurts, D. Juric, and M. Muller, International Journal of Molecular Sciences. 2018 Apr 28;19(5). pii: E1314. doi: 10.3390/ijms19051314.



**Figure 2.** Assessment of secreted markers of bone metabolism and inflammation under basal and inflammatory conditions. Osteoarthritic specimens were left untreated in osteogenic culture medium (control) or challenged with LPS. Secreted protein levels of pro-Col-1, IL-6 and MCP-1 were determined by ELISA. Adapted [reprinted] from “Novel Ex Vivo Human Osteochondral Explant Model of Knee and Spine Osteoarthritis Enables Assessment of Inflammatory and Drug Treatment Responses,” by J. Geurts, D. Juric, and M. Muller, International Journal of Molecular Sciences. 2018 Apr 28;19(5). pii: E1314. doi: 10.3390/ijms19051314.

### **4.3. Inhibition of TGF- $\beta$ Receptor Type I Signalling Modulates Bone Metabolism and Inflammatory Mediators**

Next, we sought to evaluate whether drug treatment of explanted tissues would lead to a measurable effect. As proof of concept we investigated the effects of pharmacological inhibition of TGF-beta receptor type 1 signalling, which has been described as pivotal signalling pathway in joint homeostasis and osteoarthritis. Osteochondral specimens were challenged with LPS in the presence of 10 micro M of SB-505124 and pro-Col-1, IL-6 and MCP-1 levels were determined by ELISA. Targeting of TGF-beta signalling led to significant reduction in bone metabolism, as demonstrated by a 3.4-fold reduction of pro-Col-1 secretion. Similarly, IL-6 levels were found 2.4-fold reduced. In contrast, MCP-1 expression was 1.7-fold upregulated in the presence of TGF-beta type 1 receptor inhibitor. Subgroup analysis of osteoarthritic knee and facet joint, and non-osteoarthritic cancellous bone revealed differences in treatment effects. Notably, levels of bone metabolism and inflammatory markers were significantly altered in osteoarthritic specimens, but not non-osteoarthritic controls. In addition, upregulation of MCP-1 protein expression was significant in knee, but not facet joint osteoarthritis. Together, these findings demonstrate that tissue responses of osteochondral specimens from osteoarthritic joints to inflammation and drug treatment can be monitored in an ex vivo explant model.



**Figure 3.** Assessment of secreted markers of bone metabolism and inflammation under inflammatory conditions in the presence and absence TGF-beta receptor type 1 inhibition. Osteoarthritic specimens were challenged with LPS (1 microgram per mL) and treated with 10 micro M SB-505124. Secreted protein levels of pro-Col-1, IL-6 and MCP-1 were determined by ELISA. Adapted [reprinted] from “Novel Ex Vivo Human Osteochondral Explant Model of Knee and Spine Osteoarthritis Enables Assessment of Inflammatory and Drug Treatment Responses,” by J. Geurts, D. Juric, and M. Muller, International Journal of Molecular Sciences. 2018 Apr 28;19(5). pii: E1314. doi: 10.3390/ijms19051314.

**Table 2.** Weight-normalized secreted protein levels under inflammatory conditions in the presence and absence of TGF-beta receptor type 1 signalling inhibitor.

Secreted Protein	Treatment	Total OA (n = 12)	Facet OA (n = 6)	Knee OA (n = 6)	Osteal Tissue (n = 5)
pro-Col-I (pg/mg)	LPS	1111 ± 432	1604 ± 834	618 ± 165	5460 ± 2306
	LPS + TGF-βRI	327 ± 39 ‡	278 ± 51 †	377 ± 55 †	2536 ± 1183
IL-6 (pg/mg)	LPS	652 ± 247	925 ± 471	379 ± 132	2952 ± 1620
	LPS + TGF-βRI	274 ± 82 ‡	351 ± 157 ‡	196 ± 48 †	5716 ± 4733
MCP-1 (pg/mg)	LPS	80 ± 30	125 ± 55	34 ± 3	1032 ± 680
	LPS + TGF-βRI	139 ± 40 †	200 ± 74	79 ± 7 ‡	1223 ± 819

†  $p < 0.05$ , ‡  $p < 0.01$  versus LPS group by ratio paired  $t$ -test.

*Note.* Adapted [reprinted] from “Novel Ex Vivo Human Osteochondral Explant Model of Knee and Spine Osteoarthritis Enables Assessment of Inflammatory and Drug Treatment Responses,” by J. Geurts, D. Juric, and M. Muller, International Journal of Molecular Sciences. 2018 Apr 28;19(5). pii: E1314. doi: 10.3390/ijms19051314.

## 5. Discussion

The development of a DMOAD remains an unmet clinical need in the treatment of OA in humans. Despite encouraging results from experimental OA models [19–21], the translation of preclinical studies to clinical practice has proved to be challenging. Selection of an appropriate model that accurately reflects the joint-specific pathomechanisms and clinical phenotypes observed in human disease is crucial for successful DMOAD development. Given that experimental murine models are predominantly focused on post-traumatic knee joint OA, we sought to establish a novel human ex vivo OA model based on explant culture of clinical specimens from knee and spine. Our findings showed that human osteochondral tissues could readily be cultured without appreciable loss of viability. Explanted specimens were capable of mounting an inflammatory response to a TLR4 agonist, which mimics signalling induced by endogenous ligands produced in the degenerative joint. Inhibition of TGF- $\beta$  signalling, which is pivotal in OA subchondral bone remodelling [20], reduced bone metabolism and cytokine expression. Upregulation of MCP-1 secretion was uncovered as a potential undesirable side effect of TGF- $\beta$  signalling inhibition.

To the best of our knowledge, this is the first study using intact osteochondral tissue from human OA specimens as a model for evaluating drug treatment responses. Human and mouse bone and cartilage explants have been used to investigate catabolic responses to stimulation with pro-inflammatory mediators [27,28]. Osteochondral explants from OA patients stimulated for 7 days with IL-17A and TNF- $\alpha$  showed a two- to five-fold increase of cytokine (IL-6) and chemokine (IL-8) secretion and loss of bone volume [27]. Treatment of explanted whole femoral heads with TNF- $\alpha$  and oncostatin-M for 10 days led to increased levels of Col-I resorption marker in conditioned medium [28]. Markers of bone formation, such as alkaline phosphatase, osteocalcin or pro-Col-I, were not assessed in these models. While we used a different stimulus in this study, our results confirm that an inflammatory challenge elicits an innate immune response in osteochondral tissues. Notably, pro-Col-I as a marker of bone formation and metabolism seemed unaffected under inflammatory conditions. Assessment of secreted markers of bone resorption (CTX-I or tartrate-resistant acid phosphatase) or micro-computed tomography of cancellous bone volume could provide insight whether triggering of TLR4 signalling leads to elevated bone resorption in human knee and spine OA. Interestingly,

previous histological studies demonstrated high osteoclast activity in subchondral marrow tissues of knee, but not facet joint OA [29,30].

Pleiotropic effects of growth factor and cytokine signalling in different joint tissues are important to consider in DMOAD development. TGF- $\beta$  receptor type I signalling orchestrates pathological bone formation in experimental OA [20] yet promotes chondrocyte anabolism in human and murine osteoarthritic cartilage tissues [26]. TGF- $\beta$ 1 stimulates the expression and secretion of pro-Col-I in primary human osteoarthritic osteoblasts [31]. While the effects of SB-505124 treatment on cartilage metabolism have not been investigated in this study, we uncovered upregulated MCP-1 secretion under inflammatory conditions as potential undesirable side effect of TGF- $\beta$  signal pathway inhibition.

A regulatory negative feedback loop between MCP-1 and TGF- $\beta$ 1 has previously been demonstrated in kidney tissue [32]. Conversely, a positive regulatory mechanism has been described in blood vessels [33]. Our results suggest that TGF- $\beta$  signalling in osteochondral tissues reduces MCP-1 protein levels. An important role for MCP-1 signalling in mediating monocyte recruitment, inflammation and cartilage destruction in experimental OA [12] suggest that treatment strategies based on targeting of TGF- $\beta$  signalling should be carefully evaluated. It should be noted that beneficial treatment effects were obtained by local delivery of neutralizing TGF- $\beta$  antibody into subchondral bone of a rat OA model [20].

Given the ample evidence for the involvement of crosstalk between cartilage and subchondral bone and marrow tissues in OA joints [34], it is straightforward that systemic treatment strategies targeting a specific tissue compartment need to be screened for side effects. We found increasing normalized secretion levels of pro-Col-I, IL-6 and MCP-1 in osteal tissue specimens, which contain a relatively high marrow to bone tissue fraction. It is therefore likely that expression of the aforementioned markers stems primarily from resident cells of bone (osteoblasts) and marrow (macrophages, stromal cells). Importantly, striking histological differences between clinical knee OA phenotypes and knee, spine and ankle joint OA have been described for subchondral bone marrow tissues [29,30,35,36]. The established explant model might greatly aid in evaluating whether differential inflammatory and treatment responses occur in different joints or clinical phenotypes.



Future research efforts could include the analysis of cartilage catabolic and anabolic markers such as cartilage oligomeric matrix protein, aggrecan or collagen type II fragments. In addition, it would be interesting to determine expression patterns of secreted proteins that have been described to be differentially regulated in OA tissues, such as DKK-1 and sclerostin [37,38]. Stratification of clinical specimens prior to explant culture using MRI-based assessment of joint inflammation and bone marrow lesions might aid in selectively studying clinical phenotypes [8,39].

We acknowledge some limitations of the present study. Specimens were cultured in osteogenic medium containing dexamethasone to sustain activity of bone tissues. Dexamethasone is, however, a corticosteroid with broad anti-inflammatory effects, and inflammatory tissue responses to LPS might therefore have been partially dampened. Nevertheless, we observed a clear inflammatory response, and expression of inflammatory mediators might only be increased when omitting dexamethasone from the culture medium. Furthermore, the explant model focuses on osteochondral tissue responses in an artificial setting. The role of mechanical loading, angiogenesis or crosstalk with synovial tissue and fluid is cannot be considered under the described culture conditions. The influence of synovial inflammation on osteochondral tissues could, however, be investigated by co-culture experiments or stimulation with conditioned medium. Adaptation of the explant model to a mechanical loading bioreactor commonly used for 3D-tissue engineering constructs might enable the evaluation of tissue responses under physiological and pathological joint loading conditions.

In conclusion, we have provided proof-of-concept and feasibility of an explant culture of human osteochondral clinical specimens from knee and facet joint OA for the evaluation of tissue responses to inflammation and drug treatment. Activation of LPS signalling, mimicking TLR4-induced inflammation mediated by DAMPs, resulted in an inflammatory cytokine response in osteochondral tissues. Inhibition of TGF- $\beta$  signalling, a key pathway in bone metabolism, modulated pro-Col-I secretion and differentially regulated inflammatory mediators IL-6 and MCP-1. This preclinical disease model may be valuable in defining and validating DMOAD targets in specific joints and clinical phenotypes.

## **6. Materials and methods**

### **6.1. Collection of clinical specimens**

Five knee tibial plateaus were obtained from patients undergoing total joint arthroplasty (average age  $72 \pm 5.7$  years). Six facet joints specimens were harvested by facetectomy from patients undergoing spine fusion due to lumbar spinal stenosis (average age  $74 \pm 5.9$  years). Iliac crest cancellous bone was obtained as left-over autologous bone graft material from three patients undergoing spine fusion surgery (average age  $65 \pm 8.4$  years). Written informed consent was obtained from all patients and the study protocol has been reviewed and approved by the local ethical committee.

### **6.2. Explant culture of osteochondral and cancellous bone specimens**

Specimens were processed immediately after surgical resection and gently rinsed in sterile phosphate-buffered saline (PBS) to remove blood. Degenerative facet joints, the central portion of the cartilage lesion on tibial plateaus (5 medial, one lateral) were cut in equal-sized samples (50-500 mg wet weight) with scalpel. Fragments were placed in 8 mL osteogenic culture medium in 6-well plates (alpha MEM supplemented with antibiotics, 10% fetal bovine serum, 10 mM HEPES, 4 mM L-glutamine,  $10^{-7}$  M dexamethasone, 50 micrograms of L-ascorbic acid-2-phosphate and 10 mM sodium beta-glycerophosphate pentahydrate (Sigma-Aldrich)). Specimens were cultured for one week at 37 degrees Celsius in a humidified atmosphere containing 5% carbon dioxide.

### **6.3. Inflammatory challenge and TGF- $\beta$ receptor type I inhibitor treatment**

Controls were treated with vehicle (6 microliters DMSO) and 16 microliters of PBS at days 0 and 3. To elicit an inflammatory response, specimens were treated with vehicle and 16 microliters of a 500X stock solution of lipopolysaccharide (LPS) from *Escherichia coli* O111:B4 (L2630 Sigma-Aldrich, final concentration: 1 microgram per millilitre) at days 0 and 3. For inhibition of TGF-beta receptor type 1 signalling under inflammatory conditions, specimens were treated with LPS and 10 micro M SB-505124 (Sigma-Aldrich) at day 0 and 3. At

day 7 conditioned medium was collected and stored at -80 degree Celsius until further analysis.

#### **6.4. MTT staining**

After explant culture, specimens were gently rinsed in PBS and incubated in staining solution (50 micrograms/mL MTT in sterile PBS) at 37 degrees Celsius for one hour. Samples were photographed at a digital 3D microscope (DVM6, Leica) at a magnification of 52X.

#### **6.5. Enzyme-linked immunosorbent assay (ELISA)**

Secreted protein levels of human pro-collagen-I, alpha1, interleukin-6 and monocyte chemoattractant protein 1 were determined by commercial ELISA kits (Abcam, UK, ab210966, ab178013 and ab178886) according to the manufacturer's instructions. Protein levels were normalized to the wet weight of explanted samples and expressed as pg/mg tissue.

#### **6.6. Statistical analysis**

Statistical analyses were performed using GraphPad Prism (v6.2, Graphed Software Inc., CA USA). Data followed a normal distribution and are reported as means  $\pm$ SEM. Significant differences were calculate using ratio paired t-test or one-way ANOVA. P-values less than 0.5 were considered significant.

## **7. Acknowledgements**

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## 8. References

1. Kraus, V.B.; Blanco, F.J.; Englund, M.; Karsdal, M.A.; Lohmander, L.S. Call for standardized definitions of osteoarthritis and risk stratification for clinical trials and clinical use. *Osteoarthr. Cartil.* **2015**, *23*, 1233–1241. [CrossRef] [PubMed]
2. Cho, H.J.; Morey, V.; Kang, J.Y.; Kim, K.W.; Kim, T.K. Prevalence and risk factors of spine, shoulder, hand, hip, and knee osteoarthritis in community-dwelling Koreans older than age 65 years. *Clin. Orthop. Relat. Res.* **2015**, *473*, 3307–3314. [CrossRef] [PubMed]
3. Suri, P.; Hunter, D.J.; Rainville, J.; Guermazi, A.; Katz, J.N. Presence and extent of severe facet joint osteoarthritis are associated with back pain in older adults. *Osteoarthr. Cartil.* **2013**, *21*, 1199–1206. [CrossRef] [PubMed]
4. Blagojevic, M.; Jinks, C.; Jeffery, A.; Jordan, K.P. Risk factors for onset of osteoarthritis of the knee in older adults: A systematic review and meta-analysis. *Osteoarthr. Cartil.* **2010**, *18*, 24–33. [CrossRef] [PubMed]
5. Hugle, T.; Geurts, J. What drives osteoarthritis?—Synovial versus subchondral bone pathology. *Rheumatology* **2017**, *56*, 1461–1471. [PubMed]
6. Roemer, F.W.; Kwoh, C.K.; Hannon, M.J.; Hunter, D.J.; Eckstein, F.; Fujii, T.; Boudreau, R.M.; Guermazi, A. What comes first? Multi-tissue involvement leading to radiographic osteoarthritis: Magnetic resonance imaging-based trajectory analysis over four years in the osteoarthritis initiative. *Arthritis Rheumatol.* **2015**, *67*, 2085–2096. [CrossRef] [PubMed]
7. Burr, D.B.; Gallant, M.A. Bone remodelling in osteoarthritis. *Nat. Rev. Rheumatol.* **2012**, *8*, 665–673. [CrossRef] [PubMed]
8. Felson, D.T.; Niu, J.; Neogi, T.; Goggins, J.; Nevitt, M.C.; Roemer, F.; Torner, J.; Lewis, C.E.; Guermazi, A.; Group, M.I. Synovitis and the risk of knee osteoarthritis: The most study. *Osteoarthr. Cartil.* **2016**, *24*, 458–464. [CrossRef] [PubMed]

9. De Lange-Brokaar, B.J.; Bijsterbosch, J.; Kornaat, P.R.; Yusuf, E.; Ioan-Facsinay, A.; Zuurmond, A.M.; Kroon, H.M.; Meulenbelt, I.; Bloem, J.L.; Kloppenburg, M. Radiographic progression of knee osteoarthritis is associated with MRI abnormalities in both the patellofemoral and tibiofemoral joint. *Osteoarthr. Cartil.* **2016**, *24*, 473–479. [CrossRef] [PubMed]
10. Kim, J.S.; Ali, M.H.; Wydra, F.; Li, X.; Hamilton, J.L.; An, H.S.; Cs-Szabo, G.; Andrews, S.; Moric, M.; Xiao, G.; et al. Characterization of degenerative human facet joints and facet joint capsular tissues. *Osteoarthr. Cartil.* **2015**, *23*, 2242–2251. [CrossRef] [PubMed]
11. Livshits, G.; Kalinkovich, A. Hierarchical, imbalanced pro-inflammatory cytokine networks govern the pathogenesis of chronic arthropathies. *Osteoarthr. Cartil.* **2018**, *26*, 7–17. [CrossRef] [PubMed]
12. Raghu, H.; Lepus, C.M.; Wang, Q.; Wong, H.H.; Lingampalli, N.; Oliviero, F.; Punzi, L.; Giori, N.J.; Goodman, S.B.; Chu, C.R.; et al. CCL2/CCR2, but not CCL5/CCR5, mediates monocyte recruitment, inflammation and cartilage destruction in osteoarthritis. *Ann. Rheum. Dis.* **2017**, *76*, 914–922. [CrossRef] [PubMed]
13. Schelbergen, R.F.; de Munter, W.; van den Bosch, M.H.; Lafeber, F.P.; Sloetjes, A.; Vogl, T.; Roth, J.; van den Berg, W.B.; van der Kraan, P.M.; Blom, A.B.; et al. Alarmins S100A8/S100A9 aggravate osteophyte formation in experimental osteoarthritis and predict osteophyte progression in early human symptomatic osteoarthritis. *Ann. Rheum. Dis.* **2016**, *75*, 218–225. [CrossRef] [PubMed]
14. Huang, Z.Y.; Stabler, T.; Pei, F.X.; Kraus, V.B. Both systemic and local lipopolysaccharide (LPS) burden are associated with knee osteoarthritis severity and inflammation. *Osteoarthr. Cartil.* **2016**, *24*, 1769–1775. [CrossRef] [PubMed]
15. Miller, R.E.; Belmadani, A.; Ishihara, S.; Tran, P.B.; Ren, D.; Miller, R.J.; Malfait, A.M. Damage-associated molecular patterns generated in osteoarthritis directly excite murine nociceptive neurons through toll-like receptor 4. *Arthritis Rheumatol.* **2015**, *67*, 2933–2943. [CrossRef] [PubMed]

- Van Lent, P.L.; Blom, A.B.; Schelbergen, R.F.; Sloetjes, A.; Lafeber, F.P.; Lems, W.F.; Cats, H.; Vogl, T.; Roth, J.; van denBerg, W.B. Activeinvolvementofalarmins S100A8 and S100A9 intheregulationofsynovialactivationandjointdestructionduringmouseand human osteoarthritis. *ArthritisRheum.* **2012**, *64*, 1466–1476. [CrossRef] [PubMed]
16. Johnson, C.I.; Argyle, D.J.; Clements, D.N. In vitromodels for thestudyofosteoarthritis. *Vet. J.* **2016**, *209*, 40–49. [CrossRef] [PubMed]
17. Thysen, S.; Luyten, F.P.; Lories, R.J. Targets, modelsandchallengesinosteoarthritisresearch. *Dis. ModelsMech.* **2015**, *8*, 17–30. [CrossRef] [PubMed]
18. Deshmukh, V.; Hu, H.; Barroga, C.; Bossard, C.; Kc, S.; Dellamary, L.; Stewart, J.; Chiu, K.; Ibanez, M.; Pedraza, M.; etal. A small-moleculeinhibitoroftheWntpathway (SM04690) as a potentialdiseasemodifying agent for thetreatmentofosteoarthritisoftheknee. *Osteoarthr. Cartil.* **2018**, *26*, 18–27. [CrossRef] [PubMed]
19. Zhen, G.; Wen, C.; Jia, X.; Li, Y.; Crane, J.L.; Mears, S.C.; Askin, F.B.; Frassica, F.J.; Chang, W.; Yao, J.; etal. Inhibitionof TGF- $\beta$  signalinginmesenchymalstemcellsofsubchondral bone attenuatesosteoarthritis. *Nat. Med.* **2013**, *19*, 704–712. [CrossRef] [PubMed]
20. Miller, R.E.; Tran, P.B.; Ishihara, S.; Larkin, J.; Malfait, A.M. Therapeuticeffectsofan anti-ADAMTS-5 antibody on jointdamageandmechanicalallodyniain a murine model ofosteoarthritis. *Osteoarthr. Cartil.* **2016**, *24*, 299–306. [CrossRef] [PubMed]
21. Dell’Isola, A.; Steultjens, M. Classificationofpatientswithkneeosteoarthritisinclinicalphenotypes: Data fromtheosteoarthritisinitiative. *PLoS ONE* **2018**, *13*, e0191045. [CrossRef] [PubMed]
22. Fang, H.; Beier, F. Mouse modelsofosteoarthritis: Modellingriskfactorsandassessingoutcomes. *Nat. Rev. Rheumatol.* **2014**, *10*, 413–421. [CrossRef] [PubMed]

23. Karsdal, M.A.; Michaelis, M.; Ladel, C.; Siebuhr, A.S.; Bihlet, A.R.; Andersen, J.R.; Guehring, H.; Christiansen, C.; Bay-Jensen, A.C.; Kraus, V.B. Disease-modifying treatments for osteoarthritis (DMOADS) of the knee and hip: Lessons learned from failures and opportunities for the future. *Osteoarthr. Cartil.* **2016**, *24*, 2013–2021. [CrossRef] [PubMed]
  
24. Blaney Davidson, E.N.; van Caam, A.P.; Vitters, E.L.; Bennink, M.B.; Thijssen, E.; van den Berg, W.B.; Koenders, M.I.; van Lent, P.L.; van de Loo, F.A.; van der Kraan, P.M. TGF- $\beta$  is a potent inducer of nerve growth factor in articular cartilage via the ALK5-Smad2/3 pathway. Potential role in OA related pain? *Osteoarthr. Cartil.* **2015**, *23*, 478–486. [CrossRef] [PubMed]
  
25. Blaney Davidson, E.N.; Remst, D.F.; Vitters, E.L.; van Beuningen, H.M.; Blom, A.B.; Goumans, M.J.; van den Berg, W.B.; van der Kraan, P.M. Increase in ALK1/ALK5 ratio as a cause for elevated MMP-13 expression in osteoarthritis in humans and mice. *J. Immunol.* **2009**, *182*, 7937–7945. [CrossRef] [PubMed]
  
26. Osta, B.; Roux, J.P.; Lavocat, F.; Pierre, M.; Ndongo-Thiam, N.; Boivin, G.; Miossec, P. Differential effects of IL-17a and TNF- $\alpha$  on osteoblastic differentiation of isolated synoviocytes and on bone explants from arthritis patients. *Front. Immunol.* **2015**, *6*, 151. [CrossRef] [PubMed]
  
27. Madsen, S.H.; Goettrup, A.S.; Thomsen, G.; Christensen, S.T.; Schultz, N.; Henriksen, K.; Bay-Jensen, A.C.; Karsdal, M.A. Characterization of an ex vivo femoral head model assessed by markers of bone and cartilage turnover. *Cartilage* **2011**, *2*, 265–278. [CrossRef] [PubMed]
  
28. Geurts, J.; Patel, A.; Hirschmann, M.T.; Pagenstert, G.I.; Muller-Gerbl, M.; Valderrabano, V.; Hugle, T. Elevated marrow inflammatory cells and osteoclasts in subchondral osteosclerosis in human knee osteoarthritis. *J. Orthop. Res.* **2016**, *34*, 262–269. [CrossRef] [PubMed]
  
29. Netzer, C.; Urech, K.; Hugle, T.; Benz, R.M.; Geurts, J.; Scharen, S. Characterization of subchondral bone



histopathology of facet joint osteoarthritis in lumbar spinal stenosis. *J. Orthop. Res.* **2016**, *34*, 1475–1480. [CrossRef] [PubMed]

30. Couchourel, D.; Aubry, I.; Delalandre, A.; Lavigne, M.; Martel-Pelletier, J.; Pelletier, J.P.; Lajeunesse, D. Altered mineralization of human osteoarthritic osteoblasts is attributable to abnormal type I collagen production. *Arthritis Rheum.* **2009**, *60*, 1438–1450. [CrossRef] [PubMed]
31. Lee, E.Y.; Chung, C.H.; Khoury, C.C.; Yeo, T.K.; Pyagay, P.E.; Wang, A.; Chen, S. The monocyte chemoattractant protein-1/CCR2 loop, inducible by TGF- $\beta$ , increases podocyte motility and albumin permeability. *Am. J. Physiol. Ren. Physiol* **2009**, *297*, F85–F94. [CrossRef] [PubMed]
32. Ma, J.; Wang, Q.; Fei, T.; Han, J.D.; Chen, Y.G. MCP-1 mediates TGF- $\beta$ -induced angiogenesis by stimulating vascular smooth muscle cell migration. *Blood* **2007**, *109*, 987–994. [CrossRef] [PubMed]
33. Findlay, D.M.; Kuliwaba, J.S. Bone-cartilage crosstalk: A conversation for understanding osteoarthritis. *Bone Res.* **2016**, *4*, 16028. [CrossRef] [PubMed]
34. Wyatt, L.A.; Moreton, B.J.; Mapp, P.I.; Wilson, D.; Hill, R.; Ferguson, E.; Scammell, B.E.; Walsh, D.A. Histopathological subgroups in knee osteoarthritis. *Osteoarthr. Cartil.* **2017**, *25*, 14–22. [CrossRef] [PubMed]
35. Paul, J.; Barg, A.; Kretzschmar, M.; Pagenstert, G.; Studler, U.; Hugle, T.; Wegner, N.J.; Valderrabano, V.; Geurts, J. Increased osseous  $^{99m}\text{Tc}$ -DPD uptake in end-stage ankle osteoarthritis: Correlation between aspect-CT imaging and histologic findings. *Foot Ankle Int.* **2015**, *36*, 1438–1447. [CrossRef] [PubMed]
36. Leijten, J.C.; Bos, S.D.; Landman, E.B.; Georgi, N.; Jahr, H.; Meulenbelt, I.; Post, J.N.; van Blitterswijk, C.A.; Karperien, M. GREM1, FRZB and DKK1 mRNA levels correlate with osteoarthritis and are regulated by osteoarthritis-associated factors. *Arthritis Res. Ther.* **2013**, *15*, R126. [CrossRef] [PubMed]
37. Chang, J.C.; Christiansen, B.A.; Muruges, D.K.; Sebastian, A.; Hum, N.R.; Collette, N.M.; Hatsell, S.; Economides, A.N.; Blanchette, C.D.; Loots, G.G.

SOST/sclerostin improves post traumatic osteoarthritis and inhibits MMP2/3 expression after injury. *J. Bone Miner. Res.* **2018**. [CrossRef] [PubMed]

38. Muratovic, D.; Findlay, D.M.; Cicuttini, F.M.; Wluka, A.E.; Lee, Y.R.; Kuliwaba, J.S. Bone matrix microdamage and vascular changes characterize bone marrow lesions in the subchondral bone of knee osteoarthritis. *Bone* **20189**, *108*, 193-201. [CrossRef] [PubMed]

## 9. Biography

I was born on August 17th, 1995 in Zagreb. My parents are Andrea Maria and Renato. I have a younger brother Filip studying at the University of Zagreb, Faculty of Electrical Engineering and Computing. I am fluent in English, German, Spanish and Croatian. Living and/or studying in Austria, Hungary, USA, Turkey, Spain, Croatia and Switzerland has enabled me to appreciate people of different cultures and backgrounds and to see things from other people's point of view. I started my primary education in "Walhdschule" in Vienna, Austria. I completed the International General Certificate of Secondary Education (IGCSE) at the MEF International School in Izmir, Turkey. Following that, I completed the International Baccalaureate Diploma Programme (IB) and graduated from high school at the Swiss International School in Basel, Switzerland. I enrolled into medical school in 2013 at the School of Medicine, University of Zagreb.

During my medical studies, I received the Deans Award for academic excellence. I have also been a member of "SportMEF" students sports association and leader of the medical school tennis team from 2016 until 2019. During those years, the girls team won a gold medal on the university tennis championship in Zagreb as well as on the tournament "Humanijada" in Makarska and on the national championship in Biogradna Moru. As a member of SportMEF I participated in the organization of the traditional 162 stair race at our faculty. I have also presented an oral presentation "Towards an ex vivo model of human osteoarthritis enables evaluation of osteoarthritis-disease modifying drug treatment" on the II Congress of Orthopaedic and Traumatology Surgeons with International Participation, Mostar, B&H. I also attended the COST Disaster and Bioethics Summer School, University of Birmingham, Birmingham, UK which is an event targeted at those who respond to humanitarian emergencies and disasters. There I gave an oral presentation about a case study about a meningitis epidemic in Niger. During my studies, I also attended a lot of conferences such as the Spine Experts Group Annual Meeting, Zagreb, Croatia, the 11th Croatian Congress of Plastic and Reconstructive Surgery with International Participation in Zadar, the Swiss Society of Spinal Surgery Congress, Schweizer Paraplegiker Zentrum, Nottwil, Switzerland as a last step in treatment of cervical myelopathy, Nottwil, Switzerland as well as the Croatian Student Summit, cross12, Croatian Student Summit of Biomedical Students and Young Scientists, Zagreb, Croatia.

I am a certified L1 & L2 coach with the Australian Tennis Professional Coaches Association. I have played tennis since I was 5 and have been competing in tournaments, thus gaining the ability to handle pressure. Learned that a match is over only when the last ball is played; with determination, I have won matches I was close to losing. Patience and perseverance are very often decisive in reaching a positive outcome. I have a boat skipper license, open water diver PADI international license and a windsurfing international license. I attended the Golf Education Academy in Australia as well as Theatre and Film at the Lee Strasberg Theatre and Film Institute in Los Angeles Acting at the New York Film Academy. In my free time I also enjoy to ski.

I am committed to personally strive to help others when they are most vulnerable, when they need help the most - when they are ill. In medicine I will remain humble. I have to be comfortable with recognizing the limits of my ability whilst remaining committed to lifelong learning. Medical science and technology are fascinating, every day we are reminded of the new wonders of modern medicine. A doctor's key asset is to possess an enquiring mind with the ability to absorb and draw on often diverse and sometimes conflicting scientific information. That is what I am passionate about, why I will dedicate my life to medicine. I would like my professional life to be dynamic, founded on a strong scientific background, interacting with people and working in a team with the purpose of helping others. Medicine for me is not a career, it is a way of life to which I believe I have been called. The journey of becoming a doctor, one appreciated by her patients and colleagues, is long and demanding, however fulfilling in so many unexplainable ways.