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1 **The effects of physiological and injurious hydrostatic pressure on murine *ex vivo* articular and**
2 **growth plate cartilage explants: an RNAseq study**

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14 **Abstract**

15 Chondrocytes are continuously exposed to loads placed upon them. Physiological loads are pivotal to
16 the maintenance of articular cartilage health, while abnormal loads contribute to pathological joint
17 degradation. Similarly, the growth plate cartilage is subject to various loads during growth and
18 development. Due to the high-water content of cartilage, hydrostatic pressure is considered one of the
19 main biomechanical influencers on chondrocytes and has been shown to play an important role in the
20 mechano-regulation of cartilage. Herein, we conducted RNAseq analysis of *ex vivo* hip cap (articular),
21 and metatarsal (growth plate) cartilage cultures subjected to physiological (5 MPa) and injurious (50
22 MPa) hydrostatic pressure, using the Illumina platform (n = 4 replicates). Several hundreds of genes
23 were shown to be differentially modulated by hydrostatic pressure, with the majority of these changes
24 evidenced in hip cap cartilage cultures (375 significantly upregulated and 322 downregulated in 5 MPa
25 versus control; 1022 upregulated and 724 downregulated in 50 MPa versus control). Conversely, fewer
26 genes were differentially affected by hydrostatic pressure in the metatarsal cultures (5 significantly
27 upregulated and 23 downregulated in 5 MPa versus control; 7 significantly upregulated and 19
28 downregulated in 50 MPa versus control). Using Gene Ontology annotations for Biological Processes,
29 in the hip cap data we identified a number of pathways that were modulated by both physiological and
30 injurious hydrostatic pressure. Pathways upregulated in response to 50 MPa versus control, included
31 those involved in the generation of precursor metabolites and cellular respiration. Biological processes
32 that were downregulated in this tissue included ossification, connective tissue development, and
33 chondrocyte differentiation. Collectively our data highlights the divergent chondrocyte phenotypes in

articular and growth plate cartilage. Further, we show that the magnitude of hydrostatic pressure application has distinct effects on gene expression and biological processes in hip cap cartilage explants. Finally, we identified differential expression of a number of genes that have previously been identified as osteoarthritis risk genes, including *Ctsk*, and *Chadl*. Together these data may provide potential genetic targets for future investigations in osteoarthritis research and novel therapeutics.

1 Introduction

Articular cartilage is a specialised connective tissue that covers the ends of bones in synovial joints and facilitates joint movement. It is load bearing and therefore protects underlying subchondral bone from excessive forces. The articular cartilage consists of chondrocytes which retain a stable phenotype to ensure the longevity of the tissue (1, 2). This is in contrast to the chondrocytes of the growth plate cartilage which undergo defined stages of maturation and differentiation to enable longitudinal bone growth (3).

Structurally, the articular cartilage can be divided into superficial, intermediate, and deep zones which are distinct in their organization of both the chondrocytes, surrounded by their individual pericellular matrix, and the collagen type-II and aggrecan-rich matrix (3). The articular cartilage functions to withstand physiological loading over the life-course. However, in the degenerative joint disease osteoarthritis, pathology is characterised by progressive articular cartilage degradation (4). Whilst osteoarthritis is well established to affect all tissues of the joint, the cellular and molecular mechanisms are incompletely understood (5, 6, 7). Various forms of mechanical stimuli are involved in the maintenance of the articular cartilage and thus the mechanoresponse of the chondrocyte plays an important role in the development of osteoarthritis (8, 9, 10). Compression, tensile and shear stress result in deformative loading, whereas osmotic and hydrostatic pressure induce stress without tissue or cellular deformation (8, 9, 11, 12). As a highly hydrated tissue, interstitial fluid pressurisation within the articular cartilage is considered one of the main biomechanical influencers on chondrocytes (13, 14, 15). Throughout the cartilage zones, chondrocytes are subjected and respond to a hydrostatic pressure gradient, ranging from 0.1-10 MPa, to direct matrix remodelling, chondrogenesis and chondrocyte metabolism (13, 14). However, excessive hydrostatic pressure (≥20 MPa) outside the physiological range has been shown to induce apoptosis, alter cell morphology and metabolism, reduce extracellular matrix (ECM) synthesis, induce inflammatory cytokine production, and modulate oxidative stress (16, 17, 18, 19).

In vitro, hydrostatic pressure can be applied experimentally to cells and tissues derived from both animals and humans to investigate mechanotransduction, for example in monolayer cultures (20, 21, 22, 23), micromass or pellet cultures (24, 25), 3D cell scaffolds (26, 27, 28, 29), and explant cultures (17, 22, 30, 31). The ability to provide either dynamic or continuous hydrostatic pressure, alter the magnitude and/or the duration of pressure provides an alternative approach to study the effects of mechanical stimulation (13, 32). Whilst there is little consensus within the field on the duration and pressure magnitudes in cultures, our previous meta-analysis has indicated that in human and animal-derived cells, low pressure (5 MPa) leads to anabolic responses, including elevated aggrecan expression and proteoglycan release, whereas a higher pressure (50 MPa) has a negative effect on

73 proteoglycan production (33). Therefore, it is possible to investigate the effects of hydrostatic pressure
74 at both physiological and pathophysiological levels.

75 To determine the effects of hydrostatic pressure on the molecular pathways involved in the regulation
76 of chondrocyte physiology, transcriptomic analyses are often employed to identify responsive genes.
77 Several studies in animal cells have utilised these approaches in the study of chondrocyte progenitor
78 cells, immortalised chondrocytes, and primary chondrocytes within a hydrogel; however,
79 transcriptome sequencing on *ex vivo* models has not yet been performed (21, 29, 34). Phenotypic
80 changes are often observed in cells cultured in a monolayer, with cells de-differentiating or altering
81 morphology, whereas *ex vivo* models allow examination of cells within their native environment (35).
82 Herein, the aim of this study was to perform RNAseq analysis on two murine *ex vivo* cartilage models
83 (hip cap and metatarsal) after exposure to physiological and injurious hydrostatic pressure, to examine
84 the effects of hydrostatic pressure on gene expression in two different chondrocyte phenotypes.

85 2 Methods

86 *Isolation and culture of ex vivo cartilage models*

87 All mice utilised in these studies were kept in controlled conditions at the University of Brighton and
88 all tissue isolation procedures were performed in accordance with the UK Animals (Scientific
89 Procedures) Act of 1986 and regulations set by the UK Home Office and local institutional guidelines
90 (PPL: PP3310437). Analyses were conducted blindly where possible to minimise the effects of
91 subjective bias. Animal studies were conducted in line with the ARRIVE guidelines.

92 Femoral heads were isolated from 4-week-old male C57/BL6J mice (Charles River), as previously
93 described (Fig. 1) (36). In brief, the hip joint was dislocated by applying slight pressure at the joint,
94 and the femoral cap was avulsed using forceps. At this developmental stage, the predominant
95 component of this tissue is the articular cartilage, therefore underlying subchondral bone was not
96 included. Both hip caps were pooled from each individual mouse (n=4 mice/experimental group). Hip
97 caps were cultured in Dulbecco's Modified Eagle Medium with GlutaMAX, substituted with 100 U/ml
98 penicillin, 100μg/ml streptomycin (Thermo Fisher Scientific) in a humidified atmosphere (37°C, 5%
99 CO_2).

100 Embryonic metatarsal organ cultures provide a well-established model of endochondral bone growth
101 (Fig. 1) (37). Metatarsals were isolated from E15 embryos of C57/BL6J (Charles River) mice as
102 previously described (36). Six metatarsal bones were pooled per sample (n=4 samples/experimental
103 group). Metatarsal bones were cultured in α-Minimum Essential Medium supplemented with 0.2%
104 BSA Fraction V; 1 mmol/l β-glycerophosphate (βGP); 0.05 mg/ml L-ascorbic acid phosphate; 0.05
105 mg/ml gentamicin and 1.25 μg/ml fungizone (Thermo Fisher Scientific) in a humidified atmosphere
106 (37°C, 5% CO_2).

107 *Application of hydrostatic pressure*

108 After 24 hours of culture, hips caps and metatarsals were placed into 5 ml sterile plastic syringes fitted
109 with Luer lock end caps, taking care to eliminate all air bubbles (Suppl. Fig. 1). Movement of the
110 syringe plunger allowed for equilibration of pressure between syringe contents and the pressure vessel
111 water (17). The syringes were placed in a water-filled pressure vessel at room temperature. Syringes
112 were pressurized to 0 MPa (control), 5 MPa (physiological) or 50 MPa (injurious) hydrostatic pressure
113 for 1 hour (Fig. 1; Suppl. Fig. 1). Following exposure to hydrostatic pressure, tissues were placed back
114 into the incubator and cultured for a further 24 hours in the respective media, then flash frozen at -80ºC
115 until RNA extraction.

116 *RNA extraction and sequencing*

117 Tissue (<100 mg) were defrosted on ice and 1 ml Trizol (Qiagen) was added to each sample; tissues
118 were homogenised using a mechanical disruptor, making sure to keep them cool by putting on ice every
119 15 seconds. Samples were incubated at room temperature for a minimum of 10 minutes to allow for
120 cell lysis and centrifuged at 12,000 x *g* for 15 minutes at 4°C to pellet the excess tissue, whilst retaining
121 RNA in solution. The supernatant was transferred to a clean tube and 200 µL of chloroform (Sigma)
122 added. After vigorous shaking for 20 seconds, the samples were incubated at room temperature for 3
123 minutes and then centrifuged at 12,000 x *g* for 15 minutes at 4°C to enable phase separation. The upper,
124 aqueous phase was transferred to a new tube, avoiding the interface. Following the addition of an equal
125 volume of 70% ethanol, the samples were mixed thoroughly by vortexing and total RNA purified using
126 RNeasy Mini spin columns (Qiagen), according to the manufacturer's recommendations. Purified RNA
127 was eluted in 30 µl of RNase-free water, repeating the elution twice by reapplying the elute. The
128 concentration and purity of the RNA samples were assessed using a Nanodrop One C
129 spectrophotometer (Labtech) and the quality of the RNA was assessed on a TapeStation 4200 (Agilent
130 Technologies).

131 All samples passed purity quality control checks but exhibited RNA Integrity Number (RIN) equivalent
132 values below the ideal minimum of 7 (average value 2.8). The low RIN values obtained are considered
133 typical for these explant tissue samples and suggest some partial degradation of the total RNA. DV200
134 analysis using the Agilent TapeStation 4200 software showed a percentage of fragments between 200
135 and 10000 bp ranging between 53.51-87.4% in all RNA samples. Sequencing libraries were prepared
136 using the Universal Plus™ Total RNASeq with NuQuant kit and a mouse rRNA depletion module
137 (Tecan Genomics), required for partially degraded RNA samples. Library construction strategy was
138 pair end and strand specific. Libraries were checked for quality using the TapeStation 4200, quantified,
139 normalized and sequenced on the Illumina NextSeq500 sequencer using a high-output kit (17 libraries)
140 and a mid-output kit (7 libraries).

141 *Data analysis*

142 Initial sequencing read quality control was conducted using fastqc (version 0.11.9) (38) and multiqc
143 (version 1.8) (39). Trimming was performed using TrimGalore using a minimum quality threshold of
144 20, discarding any trimmed reads shorter than 20 nucleotides. Trimmed reads were quantified using
145 kallisto quant and transcript quantifications were converted to gene level by tximport. The
146 transcriptome mapping data for all samples was imported into R for data summarisation at the gene

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147 level. The data was normalized and analysed using the DESeq2 pipeline (40). Unsupervised clustering
148 of the sample data was performed using the R packages pheatmap and pcaMethods. Significant genes
149 were identified by analysis using a model design that considered the sequencing run and strandedness
150 of the library as possible batch effects (design= ~SeqRun + Library + Condition) and applying a 5%
151 significance threshold to p-values adjusted using the Benjamini and Hochberg procedure (a
152 significance threshold referred to elsewhere in the text as padj<=0.05, or 5% FDR). For functional
153 analysis of the groups of differentially expressed genes, clusterProfiler was utilised to identify
154 significantly over-represented functional categories using a significance threshold of 5% on the
155 Benjamini and Hochberg corrected p-values (41). Annotations for the Gene Ontology (GO) Biological
156 Process (BP), from the R package org.Mm.eg.db (version 3.11.4) were used (42). Genes that were
157 significantly differentially expressed between our samples were compared to recent genome-wide
158 association studies of osteoarthritis that have identified a number of osteoarthritis risk genes (43, 44).

159 **3 Results**

160 Herein, we conducted RNAseq analysis of murine *ex vivo* hip cap (articular), and metatarsal (growth
161 plate) cartilage cultures (n=4 replicates) subjected to physiological (5 MPa) and injurious (50 MPa)
162 hydrostatic pressure. Unsupervised clustering of the gene expression data indicated a clear distinction
163 between the hip cap and metatarsal sample data, but two of the hip cap cartilage samples (*H502*
164 [exposed to 50 MPa hydrostatic pressure] and *HC4* [control, 0 MPa hydrostatic pressure]) appeared to
165 be outliers, thus were excluded from all downstream statistical analyses (Fig. 2A&B; Suppl. Fig. 2&3).

166 *Gene expression profiles of articular and growth plate cartilage*

167 Prior to differential gene expression analyses focusing on the effects of hydrostatic pressure, the gene
168 expression profiles of the two different cartilage explants were investigated to assess the genes and
169 pathways that may be differentially expressed between a transient (growth plate) and an inherently
170 stable (articular) cartilage phenotype (Suppl. Data 1). There were 2775 genes upregulated and 3368
171 genes downregulated in hip cap cartilage in comparison to metatarsal cartilage (Fig. 3A). Upregulated
172 genes with the greatest $\log_2$ fold change included ribosomal protein L9 (*Rpl9-ps4,* 39.4-fold), collagen
173 type X (*Col10a1,* 7.7-fold), and frizzled-related protein (*Frzb,* 7.2-fold) (Table 1). Downregulated
174 genes with the greatest $\log_2$ fold change included microfibrillar-associated protein 4 (*Mfap4,* 8.6-fold),
175 insulin-like growth factor binding protein 2 (*Igfbp2,* 7.3-fold) and fibroblastic growth factor 10 (*Fgf10,*
176 7.2-fold) (Table 1).

177 Next, we sought to examine whether these differentially expressed genes were enriched in particular
178 biological processes. Using annotations for GO BP, the data revealed a number of significantly
179 enriched processes, which include ossification (GO:0001503; 124 genes), bone development
180 (GO:0060348; 81 genes), cartilage development (GO:0051216; 83 genes), connective tissue
181 development (GO:0061448; 98 genes), and extracellular matrix organization (GO:0030198; 84 genes),
182 in hip cap cultures in comparison to metatarsals (Suppl. Table 1). Conversely, those that were
183 downregulated included muscle tissue development (GO:0060537; 140 genes) and muscle cell
184 differentiation (GO:0042692; 127 genes), as well as synapse organization (GO:0050808; 142 genes)
185 (Suppl. Table 1).

186 When comparing the two datasets, the hip cap data yielded many more significant changes than the
187 metatarsal data, and the greater spread of log_2 fold changes taking place in the hip cap samples suggests
188 that the hip cap cartilage explants are more responsive to changes in pressure than the metatarsal
189 explants (Fig. 3). Therefore, subsequent analyses focused on the data from the hip cap explants, with
190 the highest up- and down-regulated genes, either commonly or uniquely expressed between each group,
191 in the metatarsal data sets detailed in Suppl. Table 2 & 3.

192 *Effects of physiological and injurious hydrostatic pressure on gene expression in hip cap cartilage*
193 *explants*

194 Compared to control, there were 375 genes significantly upregulated with 5 MPa hydrostatic pressure
195 and 322 significantly downregulated in hip cap cultures (Fig. 3A, Suppl. Data 1). With injurious
196 hydrostatic pressure (50 MPa), there were 1022 significantly upregulated and 724 significantly
197 downregulated genes (Fig. 3A, Suppl. Data 1). Genes commonly modulated by hydrostatic pressure in
198 these cultures with the greatest log_2 fold changes are detailed in Suppl. Table 4. The genes uniquely
199 expressed in response to hydrostatic pressure magnitudes include *Car2* (upregulated in 5 MPa versus
200 control, 1.5-fold), *Mlip* (upregulated in 50 MPa vs control, 2.6-fold), *Tg* (downregulated in 5 MPa
201 versus control, 2.2-fold) and *Ryr3* (downregulated in 50 MPa versus control, 2.6-fold) (Table 2).

202 *GO BP enrichment analysis of differentially expressed genes*

203 Using annotations for GO BP, the data revealed significantly enriched processes including regulation
204 of cytokine production (GO:001819; 21 genes), Ras protein signal transduction (GO:007265; 20 genes)
205 and ATP metabolic processes (GO0046034; 18 genes) with 5 MPa hydrostatic pressure application
206 (Table 3, Suppl. Data 2). Conversely, process including cellular component disassembly (GO:0022411;
207 16 genes) and nuclear transport (GO0051169; 15 genes) were downregulated (Table 3, Suppl. Data 2).
208 With injurious hydrostatic pressure (50 MPa), enriched pathways included generation of precursor
209 metabolites and energy (GO:0006091; 39 genes), and cellular respiration (GO:0045333, 31 genes)
210 (Fig. 4, Table 3, Suppl. Data 2). Other upregulated GO BP relevant to the known functions of
211 chondrocytes included regulation of developmental growth (GO0048638; 33 genes), and regulation of
212 cell size (GO0008361; 21 genes) (Suppl. Data 2). Whereas those downregulated included ossification
213 (GO:0001503; 25 genes), cartilage development (GO:0051216; 18 genes), connective tissue
214 development (GO:0061448; 21 genes), and chondrocyte differentiation (GO:0002062; 17 genes)
215 (Table 3, Suppl. Data 2). Further analysis of these enriched pathways in injurious hydrostatic pressure
216 highlighted differential expression of several genes known to be involved in osteoarthritis, such as
217 *Fgf2, Ep300, Ngf, Adam9, Igfbp3*, *Sox9, Comp, Col6a1, Col6a2* and *Col11a1*.

218 *Differential expression of previously identified osteoarthritis risk genes*

219 Recent genome-wide association studies of osteoarthritis have identified a number of osteoarthritis risk
220 genes (43, 44). We therefore sought to compare whether these genes were differentially expressed in
221 response to hydrostatic pressure in our datasets (Table 4). Only one of these genes was differentially
222 expressed in our 5 MPa versus control datasets (*Wscd2,* 0.6-fold downregulation; data not shown).
223 However, with injurious (50 MPa) hydrostatic pressure application, there were 12 genes differentially

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224 expressed (Table 4). These included cathepsin K (*Ctsk,* 0.9-fold upregulation), and chondroadherin-
225 like (*Chadl,* 0.9-fold downregulation) (Table 4, Suppl. Fig. 4).

226 **4 Discussion**

227 In this study we conducted RNAseq analysis of two different *ex vivo* cartilage explants (metatarsal and
228 hip cap), to examine the effects of two magnitudes of hydrostatic pressure on gene expression. We
229 observed clear differences between the cartilage types, including the upregulation of key genes such
230 as *Frzb* and *Col10a1* in the hip cap explants. Extensive changes in gene expression were observed with
231 hydrostatic pressure in the hip cap cartilage groups, however this was to a weaker extent in the
232 metatarsal explants. Within the hip cap data set, enriched GO BP in the genes that were significantly
233 downregulated in response to injurious hydrostatic pressure (50 MPa) versus control, included those
234 involved in cartilage, bone and connective tissue development. Interestingly, these pathways were also
235 increased when comparing the hip cap to the metatarsal data, suggesting that injurious hydrostatic
236 pressure may promote a more transient-like phenotype in the hip cap cultures. This is further supported
237 by our observed enrichment of the GO BPs for developmental growth and cell size in hip caps exposed
238 to 50 MPa hydrostatic pressure. Indeed, it is well established that in osteoarthritis, the inherently stable
239 articular cartilage undergoes changes that reflect a more developmental cartilage phenotype, such as
240 that in the growth plate (3, 7). Therefore, lessons can be learnt from a better understanding of these
241 two phenotypes, and their similarities and differences in our pursuit of maintaining articular cartilage
242 health in ageing. This is of particular importance given the lack of regenerative capability of the
243 articular cartilage, thus meaning therapies for osteoarthritis remain limited (4, 7).

244 Articular cartilage covers the ends of the bones in synovial joints, and the chondrocytes within maintain
245 a stable phenotype to ensure joint health and longevity. This is in contrast to the growth plate cartilage,
246 which is more transient in nature, with chondrocytes undergoing differentiation processes which drive
247 endochondral ossification and longitudinal bone growth (3). The chondrocytes of these two
248 cartilaginous structures express different programs, further defined by our RNAseq analysis in hip cap
249 (articular) and metatarsal (growth plate) cartilage. Amongst the most differentially expressed genes in
250 our studies were *Frzb*, and *Col10a1* (both upregulated) and *Igfbp2,* and *Fgf10* (both downregulated).
251 *Col10a1* is a key determinant of chondrocyte hypertrophy, with mutant or abnormal human *Col10a1*
252 expression associated with abnormalities in this process (45, 46, 47). The increase in *Col10a1* in our
253 hip cap explants therefore suggests a greater degree of hypertrophy than in our metatarsal explants.
254 Abnormal *Col10a1* expression is a well-established feature in osteoarthritis (48, 49, 50). Similarly, two
255 SNPs in *Frzb*, an antagonist of the canonical WNT pathway, have been associated with osteoarthritis
256 (51, 52, 53). Further, in pre-clinical models, osteoarthritis severity scores are significantly higher in the
257 joints with deletion of *Frzb* compared to littermates (54). Together, our data are consistent with
258 previous studies considering the different phenotypes of these cells, thus suggesting diverging
259 phenotypes of these cell populations (55, 56, 57).

260 The high-water content of cartilage (approx. 70-80% water per wet mass) is maintained by an
261 abundance of proteoglycans in the matrix. Chondrocytes in both the growth plate and the articular
262 cartilage are subjected to a number of mechanical forces, including compressive and shear stresses,

263 during loading (9, 13). These mechanical signals then modulate biochemical activity and changes in
264 chondrocyte behaviour (22). The majority of research to date has focused on understanding
265 compressive forces on the health of the articular cartilage, however most of this force transforms to
266 hydrostatic pressure due to the interstitial fluid content of joints (14, 58). As such, it can be assumed
267 that hydrostatic pressure is the more prevalent stress to which chondrocytes are exposed. Chondrocytes
268 demonstrate an improved cartilaginous physiology when exposed to hydrostatic pressure, as indicated
269 by their increased ECM production (13). This therefore suggests that understanding the complexities
270 of hydrostatic pressure could be a potential avenue for tissue regeneration in osteoarthritis.

271 Despite the application of hydrostatic pressure being experimentally controllable, studies have varied
272 in their magnitude, style and duration of hydrostatic pressure application. Our previous meta-analysis
273 informed these factors in the experimental set up for our RNAseq study herein (33). In articular
274 cartilage during normal movement, typical hydrostatic pressure loading of 0.5–10 MPa have been
275 measured (13, 59). Our meta-analysis in 3D cultured chondrocytes confirmed that, based on aggrecan
276 gene expression data, 4–5 MPa can significantly enhance proteoglycan production (33). Conversely,
277 our meta-analysis detailed that the hydrostatic pressure magnitude of 50 MPa had a negative effect on
278 proteoglycans (33). As such, we deemed the magnitudes of physiological (5 MPa) and injurious (50
279 MPa) hydrostatic pressure to be applicable in our pursuit of understanding gene changes in our
280 explants.

281 In an RNAseq study performed on monolayer cultures, Zhu et al. used human articular chondrocytes
282 to compare hydrostatic pressure (0.1 MPa) and perfusion methods on the chondrocyte phenotype, with
283 the aim of understanding methods for reducing chondrocyte dedifferentiation in culture (60). Their
284 RNAseq analysis revealed upregulation of well-known chondrocyte genes with hydrostatic pressure
285 and conclude that a low hydrostatic pressure can be beneficial to chondrocytes (60). Further, a previous
286 microarray study examined the effects of continuous hydrostatic pressure (25 MPa) on the
287 chondrogenic ATDC5 cell line, again cultured in monolayer (21). Similarities can be observed between
288 the genes they observe to be modulated by hydrostatic pressure and ours described herein, including
289 differential expression of apoptosis-related and cartilage matrix genes (21). However, Montagne et al.
290 applied a continuous hydrostatic pressure for 24 hours, which is in comparison to our study whereby
291 we applied a single load for 1 hour and is akin to a single injurious event. Further, our examination of
292 two different magnitudes of hydrostatic pressure and in physiologically-relevant cartilage explants
293 adds further strength to our study. In addition, several genes known to play a key role in progression
294 of osteoarthritis (e.g., *Fgf2, Ep300, Ngf, Adam9, Igfbp3*, *Sox9, Comp, Col6a1, Col6a2* and *Col11a1*)
295 were modulated in our injurious hydrostatic pressure hip cap datasets, thereby validating this approach.

296 Overall, our results seem to indicate osteoarthritic-like effects of injurious hydrostatic pressure on our
297 hip cap cartilage explants. Among the modulated genes identified in our study, several genes which
298 have been identified as osteoarthritis risk genes from recent GWAS studies were differentially
299 expressed, however verification of these by *in situ* hybridisation or RT-qPCR would be beneficial (43,
300 44). There was only one gene (*Wscd2*, WSC Domain-Containing Protein 2) modulated in the 5 MPa
301 versus control dataset, with the majority being in the 50 MPa comparison. Interestingly, *Wscd2* has
302 previously been identified as an osteocyte transcriptome signature gene and downregulated in murine

303 bone with ageing, although its role in cartilage has, to our knowledge, not yet fully been defined (61,
304 62).

305 Of these risk genes modulated by 50 MPa hydrostatic pressure, the gene that underwent the highest
306 fold upregulation was cathepsin K (*Ctsk*), a protein expressed by osteoclasts used for collagen
307 degradation (63). This finding is consistent with the previous microarray study by Montague et al. in
308 which *Ctsk* was found to be strongly induced following the exposure of hydrostatic pressure for 4 hours
309 (21). Indeed, *Ctsk* has been shown to be overexpressed in the articular cartilage and subchondral bone
310 in osteoarthritis (64, 65). Further, *Ctsk* deletion in a murine surgical osteoarthritis model
311 (destabilisation of the medial meniscus) protected against disease progression (66), as did
312 pharmacological treatment with a cathepsin K inhibitor (SB-553484) in a canine model (67). Pre-
313 clinical findings have been translated to clinical trials with the selective cathepsin K inhibitor MIV-
314 711 reducing bone and cartilage disease progression in individuals with symptomatic, radiographic
315 knee osteoarthritis (68).

316 *Chadl,* which encodes for chondroadherin-like protein, plays a role in collagen binding and in the
317 negative regulation of chondrocyte (69). In our studies, its expression underwent the highest fold
318 downregulation with 50 MPa hydrostatic pressure. This is consistent with a previous RNAseq study
319 which examined the subchondral bone of patients who underwent total joint replacement due to
320 osteoarthritis (70). In this study both *Chadl* and *Il11*, also identified in our studies, were identified as
321 the most consistently differentially expressed genes and thus have the potential to be targeted for
322 clinical therapies.

323 Whilst several ion channels known to be involved in chondrocyte mechanotransduction (e.g., *Piezo1*,
324 *Trpv4*, *Trpv5*) (9) were unchanged in our datasets, upregulation of *Piezo2* and downregulation of
325 *Trpm4* was observed in hip caps exposed to both magnitudes of hydrostatic pressure (Suppl. Data 1).
326 Interestingly, reliable detection of *Piezo2* transcripts in primary murine chondrocytes appears to be
327 conflicting in the literature (71, 72). *Trpm4* has been identified in cartilage samples from osteoarthritic
328 patients (73), however its role in cartilage mechanotransduction is unclear. Downregulation of *Trpm5*
329 and *P2rx7* was only observed in hip caps exposed to 5 MPa compared to control (Suppl. Data 1). This
330 suggests that whilst our *ex vivo* models are sensitive to some changes in ion channel expression with
331 hydrostatic pressure, other mechanisms may exist.

332 Our study is unique in using two different cartilage explants, both of which offer a physiological model
333 system. We have also applied hydrostatic pressure at magnitudes based on findings from our previous
334 meta-analysis to ensure these are representative of both physiological and injurious load (33).
335 However, we do recognise the limitation in our sample size presented herein. Therefore, the biological
336 interpretation of our findings should be considered appropriately, with the need for a more detailed
337 consideration of the differences observed. For example, it would be pertinent to use a temporal
338 approach to the application of hydrostatic pressure as in this study we applied a single load for 1 hour
339 and is akin to a single injurious event, rather than the continual degradation seen in osteoarthritis. It
340 would also be of further interest to utilise cartilage from an osteoarthritis model (e.g., STR/ort mouse),
341 or ultimately from human samples, to both validate our results here, and also examine the effects of

hydrostatic pressure on gene expression in disease pathology. Despite these limitations, the current study was able to statistically differentiate the effects of hydrostatic pressure on chondrocytes.

In conclusion, we identified distinct differential gene expression signatures in hip cap and metatarsal cartilage explants, indicative of the divergent phenotypes of their residing chondrocytes. Our RNAseq studies examining the cartilage response to hydrostatic pressure provided evidence for injurious hydrostatic pressure to be associated with decreases in processes including cartilage development and chondrocyte differentiation. Together this informs on the potential benefits of hydrostatic pressure in cartilage tissue engineering strategies, which need to carefully consider the magnitude of application and the effects on gene expression. Further, we identified the differential expression of a number of genes that have previously been identified as osteoarthritis risk genes, including *Ctsk* and *Chadl*, further highlighting their potential as therapeutic targets. These data will therefore contribute to a better understanding of the role of hydrostatic pressure and the chondrocyte phenotype in health and osteoarthritis.

5 Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

6 Author Contributions

Study design: KAS, PGB, GB; Analysis and interpretation of the data; all authors; Drafting the manuscript: LEB, AS, KAS; Editing and approving final version: all authors.

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9 Data Availability Statement

The RNA sequencing data are available from NCBI Gene Expression Omnibus (https://www.ncbi.nlm.nih.gov/geo) under accession number GSE234112: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE234112

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11 Figure legends

Figure 1: Schematic of experimental design. E15 metatarsal bones and 4-week-old hip cap cartilage explants were subjected to hydrostatic pressure (0-50 MPa) for 1 hour. After 24 hours, RNA was extracted and RNAseq and downstream analyses conducted. Created with BioRender.com.

Figure 2: Unsupervised clustering of samples based on their DESeq2 normalised gene-level counts. **(A)** Heat map of inter-sample Euclidean distances, where darker blue colours indicate closer similarity. **(B)** Principal components analysis. Samples are labelled as M (metatarsal) and H (hip cap), followed by C (control – 0 MPa), 5 (5 MPa) or 50 (50 MPa) and replicate number (1-4).

Figure 3: Summary of genes identified as significantly differently expressed between the main sample conditions of interest (DESeq2 padj<=0.05 (5% FDR)). **(A)** Numbers of significant genes (padj<=0.05) in each comparison. Sig. Down indicates genes down-regulated in the first condition listed in the comparison column relative to the second, while Sig. Up indicates those up-regulated in the first condition. **(B)** Overlap between the significant genes identified in each hip cap (Hip) and metatarsal (Mtarsal) cartilage explant group.

Figure 4: GO BP categories in 50 MPa versus control hip cap datasets. **(A)** Pathways enriched in the genes significantly up in the 50 MPa versus control comparisons. **(B)** Pathways enriched in the genes significantly down in the 50 MPa versus control comparisons.

601 **12 Tables**

602 **Table 1:** Top 10 genes with highest upregulation and top 10 genes with highest downregulation in
603 the hip cap versus metatarsal RNAseq datasets.

604

	Gene Name	Log_2 Fold Change	Adjusted *p* value
Top 10 genes with highest upregulation	*Rpl9-ps4*	39.41761815	6.53953E-15
	Gm10925	23.98054338	1.87468E-05
	Gm22969	21.45491466	5.33958E-06
	Mif-ps4	14.34623609	1.37344E-05
	Col10a1	7.736184458	3.71322E-08
	Serpina1d	7.646536307	5.54893E-13
	Frzb	7.219881296	2.58347E-50
	Cytl1	7.218161967	2.07064E-45
	Gpx3	7.055584365	1.53747E-28
	Clec3a	7.031246207	4.9778E-14
Top 10 genes with highest downregulation	*Mfap4*	-8.564081588	4.07328E-34
	Xist	-8.407700367	5.36182E-12
	Actc1	-8.087469882	1.48585E-10
	Hoxd13	-7.737458742	1.44143E-30
	Myh3	-7.539165841	9.66177E-24
	Kera	-7.465016823	2.52732E-12
	Igfbp2	-7.34202703	1.197E-12
	Crabp1	-7.311799886	8.05824E-13
	Fgf10	-7.176158119	4.15686E-11
	Ptn	-7.059869481	1.53489E-97

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608 **Table 2:** Top 10 genes with highest upregulation and greatest downregulation that are uniquely
609 expressed in 5 MPa versus control and 50 MPa versus control in the hip cap RNAseq datasets.

	5 MPa vs Control			50 MPa vs Control		
	Gene Name	**Log_2 Fold Change**	**Adjusted *p* value**	**Gene Name**	**Log_2 Fold Change**	**Adjusted *p* value**
Top 10 genes with highest upregulation	*Abhd15*	3.22895414	0.000519	*Gm2451*	18.04382867	1.98582E-05
	Car2	1.466531813	0.008049	*H2ac23*	15.91830736	0.000179818
	Gm45665	1.436157039	0.00787	*Gm9973*	3.311225437	5.25837E-08
	Olfr1380	1.18544216	0.033261	*Gm48942*	3.201576037	3.37509E-06
	Mpp5	1.14918265	0.000377	*Gm29408*	3.061996469	4.83774E-06
	Gm9962	1.142757676	0.013066	*Mlip*	2.567534817	0.000385784
	Fpr1	1.061193105	0.016204	*Mmp12*	2.501682447	3.10048E-05
	Atp5g2	1.039789631	0.00418	*Abcd2*	2.312011543	0.001575342
	Fam81a	1.004756565	0.011008	*Ywhaq-ps3*	2.243694849	0.046074893
	Alg8	0.977342633	0.001856	*mt-Nd6*	2.195320542	0.001507966
Top 10 genes with highest downregulation	*Rps18-ps6*	-3.33818292	0.005493	*Gm44732*	-4.24186258	0.000124443
	Gm23680	-3.158700551	0.000322	*Gm16479*	-3.327634912	0.000538299
	Gm9968	-3.054024647	0.000146	*Ryr3*	-2.596299426	0.002870783
	Gm24514	-2.45810073	1.17E-06	*Gm3625*	-2.176518378	1.83627E-05
	Tg	-2.18679795	0.001011	*Pla2g2c*	-2.166700674	0.046074893
	Gm25682	-1.92383803	0.003421	*Gm8249*	-1.940743018	0.002267981
	Gm42715	-1.658526363	0.000376	*Serpina1a*	-1.854010245	0.014092563

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	Adgrb1	-1.380005988	0.005428	*H2-M5*	-1.718905493	0.003710939
	Gm26822	-1.305512292	0.001159	*Gm15807*	-1.69503207	0.003989171
	Rap1gap2	-1.246295417	0.012053	*Serpina1d*	-1.649670074	0.000777013

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611

612 **Table 3:** Annotations for the Gene Ontology (GO) Biological Process (BP) for genes that are
613 differentially expressed in 5 MPa versus control and 50 MPa versus control in the hip cap RNAseq
614 datasets.

5 MPa vs Control				
	ID	**Description**	**No. genes**	**Adjusted *p* value**
Top 10 upregulated GOBP	GO:0001819	positive regulation of cytokine production	21	1.38E-06
	GO:0007265	Ras protein signal transduction	20	1.50E-06
	GO:0046034	ATP metabolic process	18	1.05E-08
	GO:0045333	cellular respiration	17	1.39E-10
	GO:0015980	energy derivation by oxidation of organic compounds	17	1.11E-07
	GO:0022904	respiratory electron transport chain	13	8.59E-11
	GO:0022900	electron transport chain	13	1.61E-10
	GO:0042773	ATP synthesis coupled electron transport	12	3.79E-11
	GO:0006119	oxidative phosphorylation	12	9.95E-09
	GO:0042775	mitochondrial ATP synthesis coupled electron transport	9	8.31E-08
Top 10 downregulated GOBP	GO:0022411	cellular component disassembly	16	3.92E-06
	GO:0006913	nucleocytoplasmic transport	15	1.31E-06
	GO:0051169	nuclear transport	15	1.31E-06
	GO:0033157	regulation of intracellular protein transport	11	3.94E-05
	GO:0051168	nuclear export	10	3.58E-06
	GO:0015931	nucleobase-containing compound transport	10	4.64E-05
	GO:0006611	protein export from nucleus	9	1.07E-05

	ID	Description	No. genes	Adjusted p value
	GO:0034453	microtubule anchoring	5	1.01E-05
	GO:0018023	peptidyl-lysine trimethylation	5	0.000157
	GO:0034454	microtubule anchoring at centrosome	3	0.000166
50 MPa vs Control				
	ID	**Description**	**No. genes**	**Adjusted *p* value**
Top 10 upregulated GOBP	GO:0006091	generation of precursor metabolites and energy	39	5.16E-09
	GO:0015980	energy derivation by oxidation of organic compounds	34	2.82E-11
	GO:0045333	cellular respiration	31	1.64E-14
	GO:0046034	ATP metabolic process	29	1.28E-08
	GO:0022904	respiratory electron transport chain	22	4.28E-14
	GO:0022900	electron transport chain	22	1.27E-13
	GO:0006119	oxidative phosphorylation	20	1.47E-10
	GO:0042773	ATP synthesis coupled electron transport	19	1.49E-13
	GO:0042775	mitochondrial ATP synthesis coupled electron transport	16	7.34E-11
	GO:0009060	aerobic respiration	14	2.61E-07
Top 10 downregulated GOBP	GO:0001503	ossification	25	5.11E-05
	GO:0032386	regulation of intracellular transport	23	6.10E-05
	GO:0061448	connective tissue development	21	3.88E-05
	GO:0006913	nucleocytoplasmic transport	21	4.75E-05
	GO:0051169	nuclear transport	21	4.75E-05
	GO:0048193	Golgi vesicle transport	20	3.78E-05
	GO:0051216	cartilage development	18	2.02E-05
	GO:0002062	chondrocyte differentiation	17	2.76E-08
	GO:0051168	nuclear export	13	8.25E-05
	GO:1903909	regulation of receptor clustering	5	9.33E-05

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618 **Table 4:** Differential expression of osteoarthritis risk genes identified in recent genome-wide
619 association studies in response to injurious hydrostatic pressure (50 MPa) versus control in our hip
620 cap datasets.

	Gene Name	**Log$_2$ Fold Change**	**Adjusted *p* value**
Upregulated genes	*Ctsk*	0.852667719	0.016121811
	Il11	0.750247415	0.036698304
	Sbno1	0.740095825	0.005159578
	Aldh1a2	0.648544373	0.037375403
Downregulated genes	*Chadl*	-0.904205798	0.009053645
	Apoe	-0.899057162	0.001558965
	Mn1	-0.790048116	0.020283481
	Pfkm	-0.736351338	0.017447093
	Megf8	-0.647300732	0.001249084
	Fto	-0.492563212	0.035580843
	Vgll4	-0.490894811	0.049748082
	Smg6	-0.47338466	0.043509481

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