**Title:**

*Unravelling the BCP crystal-dependent chondrocyte protein secretome; a role for TGF-β signaling*

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Abstract

*Objective:*

BCP crystals play an active role in the progression of osteoarthritis. Here, we investigate the changes in the protein secretome of human OA articular chondrocytes as a result of BCP stimulation using two unbiased proteomic analysis methods.

*Design:*

BCP crystals were produced by alkaline hydrolysis of brushite. Isolated human OA articular chondrocytes were stimulated with BCP crystals and examined by RT-qPCR and ELISA. Forty-eight hours conditioned media were analyzed by label-free LC-MS/MS and a cytokine array. The activity of BCP dependent TGF-β signaling was analysed by RT-qPCR and luciferase reporter assays. The molecular consequences regarding pro-inflammatory factor and catabolic changes of the BCP-dependent TGF-β signaling were investigated using specific pathway inhibitors.

*Results:*

Biological activity of the synthesized BCP crystals was confirmed by IL-6 expression and secretion upon stimulation of human articular chondrocytes. Concomitant induction of catabolic gene expression was observed. Analysis of conditioned media revealed a complex and diverse response with a large number of proteins involved in TGF-β signaling, both in activation of latent TGF-β and TGF-β superfamily members. Activity of this BCP driven TGF-β signaling was confirmed by increased activity of expression of TGF-β target genes and SBE luciferase reporter. Inhibition of BCP driven TGF-β signaling resulted in decreased IL-6 expression and secretion with a moderate effect on catabolic gene expression.

*Conclusions:*

BCP crystal stimulation of human OA articular chondrocytes results in a complex and diverse protein secretome response, with a major role for BCP driven TGF-β signaling in creating a pro-inflammatory environment.

Introduction

Osteoarthritis (OA) is a complex degenerative joint disease associated with ageing and involving all tissues of the affected joint. Clinical manifestations include, but are not limited to, cartilage degeneration and the presence of calcium-containing crystals1. The physiological function of articular cartilage is to provide transmission of loads with low friction. An important prerequisite for this function is the composition of the cartilage extracellular matrix (ECM). In a healthy knee joint, the protein composition of the cartilage ECM is dominated by collagen type II and aggrecan. In OA, this protein composition is critically altered with the expression of collagen type X and collagen type I. This marks the differentiation of OA chondrocytes towards hypertrophic-2 and fibrochondrocytes3. This alteration in the cartilage ECM composition with increasing quantities of collagen type I and X facilitates the formation of calcium-containing crystals (CaC) 4.

Two different types of CaC have been demonstrated to be present in OA joints: calcium pyrophosphate dihydrate (CPP) and basic calcium phosphate (BCP) crystals5. Up to 20% of OA affected joints contain CPP crystals6. In contrast, BCP crystals have a higher prevalence With between 30 and 60% of knee OA patients having BCP crystals present in their synovial fluid7. In articular cartilage this prevalence increases to 100% at the time of total knee arthroplasty8.

The general consensus regarding the role of CaC in OA remains controversial. While the presence of these crystals has been known for several decades, they were generally considered bystanders or degradation products of the underlying subchondral bone9. However, clinical and experimental data support a critical contribution of CaC in the pathobiology of OA. Injection of BCP crystals into murine knee joints resulted in synovial inflammation accompanied by cartilage degradation10. Compelling evidence of an active role of CaC in OA is further provided by a range of *in vitro* cell studies. In OA synovial fibroblasts, BCP exposure increased expression of *COX-1* and *COX-2*11. Also, BCP-dependent induction of matrix metalloproteinases MMP-1, -3 and -13 has been reported in synovial fibroblasts12, 13. In primary macrophages, increased secretion of IL-1β, S100A8, S100A12 and MMP-1 was demonstrated14. Studies investigating the response of articular chondrocytes to BCP crystals revealed increased expression of *MMP-13*15, induction of hypertrophic differentiation by sequestering of Wnt3A16, and increased secretion of IL-617.

Despite the fact that it is becoming increasingly clear that BCP crystals play a role in OA, a comprehensive understanding of the BCP crystal-induced secretome response of articular chondrocytes is lacking. Therefore, we aimed to delineate the changes in the chondrocyte protein secretome in response to BCP crystals by applying label-free LC-MS/MS and a cytokine antibody array to determine the mechanistic responses.

# Materials and methods

**BCP crystal synthesis**

BCP crystals were produced by alkaline hydrolysis of brushite 18. Chemical properties of the synthesized BCP crystals were analyzed with a Frontier FT-IR / NIR spectrometer (Perkin Elmer). BCP crystals were sterilized at 120⁰C for 15 minutes. Prior to use, BCP crystals were resuspended in DMEM/F12 at a concentration of 5 mg/ml and briefly sonicated. Final concentration of BCP crystals in all experiments was 50 µg/ml.

**Chondrocyte isolation and culture**

Articular cartilage was obtained from patients undergoing total knee arthroplasty at Maastricht UMC+. Informed consent and ethical approval were obtained by the Local ethical committee with approval ID: METC 2017-0183. Isolation of chondrocytes was performed as previously described19 and cultured in DMEM/F12 supplemented (Gibco) with 10% Fetal calf serum (FCS) and 1% antibiotic/antimycotic (Gibco). Unless indicated otherwise, a pool consisting of 12 HAC donors at passage 2 was used. OA HACs were seeded at 30.000 cells/cm2 and allowed to attach for 18 hours before start of an experiment. Inhibitors used 5µM SB-505124 (ALK4/5/7i; Selleck Chemicals) 20-22, 0.5 µM (5Z)-7-Oxozeaenol (TAK-1i; Merck Millipore) 20, 21, 23, and 10 µM parthenolide (NF-κBi 2; Sigma) 24.

**RNA isolation and RT-qPCR**

Chondrocytes were lysed with TRIzol™ Reagent (ThermoFisher Scientific). After phase separation, the aqueous phase was precipitated with isopropanol overnight at -20⁰C. The precipitate was centrifuged for 60 minutes at 20.000 x g. RNA pellets were washed with 80% ethanol and resuspended in RNAse free MQ water. RNA concentrations were determined by Nanodrop™ One (ThermoFisher Scientific). Complementary DNA was reverse transcribed from 250ng RNA using random hexamers and M-MLV reverse transcriptase (Promega) in a Biometra TRIO (Analytikjena) thermocycler with the following protocol: 6 minutes at 72⁰C followed by 1 hour and 5 minutes at 37⁰C and 5 minutes at 95⁰C. RT-qPCR was done with Takyon NO ROX SYBR MasterMix dTTP blue (Eurogentec) and all reactions contained 6 ng of cDNA, 20 μM forward primer and 20 μM reverse primer in a reaction volume of 15 μl. cDNA was amplified in a Bio-Rad CXF96 Real-Time PCR Detection system according to the following protocol: 10 minutes denaturation at 95⁰C prior to 40 amplification cycles consisting of 15 seconds at 95⁰C and 1 minute at 60⁰C. RT-qPCR primers used in this study are presented in supplementary Table 1. Gene expression levels were quantified with the standard curve method and normalized to the reference gene *PPIA*.

**Enzyme-linked immune sorbent Assay**

Secreted protein levels in the culture medium were determined by enzyme-linked immune sorbent assay (ELISA). Protein concentration was determined for hIL-6 (R&D), hCXCL8 (R&D), total hMMP-1 (R&D), hTIMP-1 (R&D) and hActivin A (R&D). All ELISA’s were performed according to manufacturer’s protocol. Absorbance was measured on a MultiSkan™ FC Microplate Photometer (ThermoFisher Scientific).

**Preparation of human OA chondrocyte conditioned media**

Fourteen patients included for analysis were selected based on gender, age and BMI. Patient population comprised six females (age = 72.8 years ± 6.6; BMI = 31.6 kg/m2 ± 4.08) and eight males (age = 70.5 years ± 6.4; BMI = 30.3 kg/m2 ± 2.17). Chondrocytes of the individual donors were seeded at a density of 30.000 cells/cm2 in phenol-red free DMEM/F12 supplemented with Glutamax (Gibco), 10% FCS and 1% antibiotic/antimycotic (Gibco). After cell adherence, chondrocytes were washed twice with pre-warmed 0.9% NaCl and stimulated in phenol red-free DMEM/F12 supplemented with Glutamax (Gibco), 0.1% FCS and 1% antibiotic/antimycotic (Gibco) with or without 50 μg/ml BCP crystals. After 48 hours, medium was harvested and centrifuged at 265 x g for 5 minutes at 4⁰C. Supernatant was removed and centrifuged again at 20.000 x g for 10 minutes at 4⁰C. Proteolytic degradation in the remaining supernatant was inhibited by addition of 1x cOmplete™ Mini, EDTA-free Protease inhibitor cocktail (Roche). Samples were stored at -80⁰C in Protein LoBind® tubes (Eppendorf) until further processing. Protein concentration in the medium was determined using a bicinchoninic acid assay.

**Liquid chromatography tandem mass spectrometry and label-free quantification**

Twenty-five μg of protein was trypsin digested for fourteen samples on 10 μl Strataclean (Agilent, Genomics, UK) as described previously25. For LC-MS/MS, 500 ng of each digest was used, using a 2-hour gradient. A QExactive HF quadrupole-Orbitrap mass spectrometer coupled to a Dionex Ultimate 3000 RSLC nano-liquid chromatograph (Hemel Hempstead, UK) was used for data-dependent analyses as previously described26.

The raw files of the acquired spectra were analysed using label-free quantification using ProgenesisQI™ software (Waters, Manchester, UK)27 which aligns the files and then peak picks for quantification by peptide abundance. The top five spectra for each feature were exported from ProgenesisQI™ and utilized for peptide identification with a Mascot server (Version 2.6.2). We searched against the Unihuman database using carbamidomethyl cysteine as a fixed modification and methionine, proline and lysine oxidation as variable modifications, peptide mass tolerance of 10 ppm and MSMS tolerance of 0.01 Da. Proteomic data has been deposited in the PRIDE ProteomeXchange and can be accessed using the identifier XXXXX.

**Antibody array**

Soluble proteins in the conditioned media were analyzed with the Human Cytokine array L507 (AAH-BLG-1, Raybiotech). Conditioned media from BCP and unstimulated cells were pooled (n=14), creating two samples. Samples were measured in duplicate. Analysis was performed by tebubio. The average intensity of the signal was calculated and used for subsequent analyses.

**Culture and lentiviral transduction of SW1353 cells**

Production of the lentiviral constructs was done as previously described 28. Viral supernatants were generated in HEK293T cells. Lentiviral transduction of SW1353 cells was performed in presence of 8 μg/ml polybrene. Selection was performed with puromycin (2 μg/ml, Sigma-Aldrich). After establishing successful transduction, lentiviral SBE and NF-κB SW1353 reporter lines were seeded at a density of 80.000 cells/cm2 in DMEM/F12 containing 0.5% FCS and 1% antibiotic/antimycotic. Reporter cells were stimulated with freshly generated OA HAC control or BCP conditioned medium. After 6 hours, cells were lysed with cell culture lysis reagent (Promega), collected and spun down at 15000rpm for 10 minutes. Luminescence was measured after addition of Nano-Glo Reagent (1:1 (v/v); Promega) utilising the Tristar2 LB942 multi-mode plate reader (Berthold Technologies).

**Statistical analysis**

Statistical significance for single comparisons was determined by unpaired or paired Student’s t-test. One-way ANOVA with Dunnett’s post-hoc test was used for multiple comparisons (Graphpad Prism 8.3.0). Results were considered significant when p. value ≤ 0.05.

Results

**BCP crystal stimulation results in pro-inflammatory and catabolic gene expression in human articular chondrocytes**

First, we synthesized BCP crystals by alkaline hydrolysis of brushite. Analysis by Fourier transformed infrared spectroscopy showed close resemblance with the structure of BCP crystals found in OA synovial fluid (Supplemental figure 1). Previous studies have shown BCP-driven IL-6 secretion by human cartilage explants17, therefore we determined the *IL-6* expression and secretion of human OA chondrocytes treated with BCP crystals. Human OA articular chondrocytes were stimulated with 50 µg/ml BCP crystals for 24 and 48 hours. *IL-6* expression was increased 7.25-fold after 24 hours and 5.00-fold after 48 hours in response to BCP crystals (Figure 1A). Moreover, secreted levels of IL-6 were increased after BCP exposure. Twenty-four hour stimulation resulted in 1800 pg/ml and 2400 pg/ml at 48 hours after BCP stimulation

Degradation of ECM components by MMPs has been well described in OA. To investigate the potential contribution of BCP crystals in this process, we determined gene expression levels of *COL2A1*, *ACAN*, *MMP-1*, *MMP-3* and *MMP-13*. Gene expression analyses demonstrated a significant decrease of *COL2A1* and *ACAN* gene expression at both 24- and 48-hour following chondrocyte BCP crystal exposure (Figure 1B). In contrast, BCP crystal stimulation of chondrocytes resulted in increased expression of *MMP-1* and *MMP-3* at both time points (Figure 1C). We observed no alteration in *MMP-13* gene expression when human chondrocytes were exposed to BCP crystals (data not shown). BCP-dependent expression of MMP-1 and MMP-3, and MMP-13 in human fibroblasts cells was reported previously15, 29, 30. Overall, we confirmed the pro-inflammatory and catabolic nature of our synthesized BCP crystals on primary human OA articular chondrocytes.

**Label-free LC-MS/MS analysis of the BCP-dependent chondrocyte protein secretome**

To acquire a comprehensive understanding of the protein secretome of OA HACs in response to BCP crystals, we applied label-free LC-MS/MS proteomics. OA HACs from fourteen individual knee OA donors were individually stimulated with BCP crystals for 48 hours and conditioned media were collected. As a confirmation of OA HAC donor responsiveness to BCP crystal stimulation, IL-6 secretion by the individual HAC donors was determined. Nine out of 14 patients showed significantly increased IL-6 secretion in response to BCP crystal stimulation (Figure 2A), while others showed an average increase that was not significantly different from control. As a representation of the OA patient variability, all individual conditioned media were included in this analysis and analyzed by label-free LC-MS/MS separately. In total, we identified 158 different proteins in the secretome. We found eighteen differentially expressed (DE) proteins, of which fifteen were upregulated in response to BCP crystal stimulation and three were downregulated (Figure 2B; Table 1.) The protein with the highest fold upregulation in response to BCP stimulation was MMP-1 (7.14-fold). The most downregulated protein was keratin type II, which decreased 3.85-fold. The majority of the DE proteins have roles in cartilage ECM composition and its remodeling, such as COL6A3, COMP, MMP-1, and TIMP-1 (Figure 2C). The observed significant increases in MMP-1 and TIMP-1 were validated by ELISA (Figure 2D).

In addition to the detection of proteins involved in ECM composition and its reorganisation, three (plasminogen, annexin A2, and gelsolin) out of the fifteen upregulated proteins (indicated by \* in Table 1.) can be linked to TGF-β release and signaling. Plasminogen, the zymogen form of plasmin, and Annexin A2, are both involved in the release of TGF-β from the latent-transforming growth factor beta-binding protein31, 32, while gelsolin promotes the expression of TGF-β in articular chondrocytes33.

**Cytokine array analysis reveals a complex change in the chondrocyte protein secretome following BCP crystal stimulation**

To be able to determine the BCP-dependent changes in the OA HAC protein secretome for smaller soluble proteins in a complementary, LC-MS/MS-independent manner, an antibody array was used. Analysis of conditioned medium of either control or BCP-stimulated chondrocytes revealed 35 DE proteins between the BCP condition and control (Figure 3A; Table 2). The largest increase was detected for MMP-13 (7.49-fold compared to control). Other significantly upregulated proteins in the BCP-dependent chondrocyte secretome included members of the TGF-β superfamily, FGF protein family, chemokines and chemokine receptors, TNF family members and receptors, MMP’s, cytokines, and adipokines (Figure 3B). A selection of the significantly upregulated proteins was validated in either the individual HAC donors or in the pools of conditioned media. Analysis of VEGF in the secretomes of individual donors confirmed its significant increase after BCP stimulation (Figure 3C). Cytokine array results were also validated by ELISA for Activin A, MMP-1 and TIMP-1 (Figure 3D). The DE proteins detected by these cytokine array measurements revealed a limited overlap with the DE proteins detected by LC-MS/MS. Comparison of the total identified proteins in both datasets, showed overlap of 14 proteins (Figure 3E). The similarity in the DE proteins was observed for two proteins: MMP-1 and TIMP-1. This data augments the characterisation of BCP-driven changes in the OA HAC protein secretomes by revealing additional soluble proteins.

**BCP crystal stimulation results in increased expression of TGF-β target genes and activates NF-κB and canonical TGF-β signaling**

The role of TGF-β and its signaling have been extensively studied in OA34. Among the many effects of increased TGF-β activity in OA is the contribution to chronic inflammation and the catabolic phenotype in human OA articular chondrocytes35, 36. Differentially expressed proteins detected by the label-free LC-MS/MS (plasminogen, annexin A2 and gelsolin) and cytokine array (GDF1, Activin A, Chordin-like 1, Activin RIIA, BMP-4 and TGF-β2) suggest the involvement of TGF-β dependent cellular signaling in the BCP crystal response of HACs. Therefore, we aimed to investigate the activation of TGF-β signaling in response to BCP crystal stimulation in OA HACs. BCP crystal stimulation of OA HACS resulted in increased expression of TGF-β target genes *PAI-1*, *SMAD-7* and *ID-1* (Figure 4A). Expression of *PAI-1* increased after 24- and 48-hours exposure to BCP crystals, while *SMAD-7* and *ID-1* only specifically increased after 24 hours (Figure 4A). TGF-β signaling occurs via different intracellular routes, for example via SMAD2/3 (canonical)37, TAK-1 mediated activation of JNK, P38 and NF-κB (non-canonical)38. To investigate whether the BCP-dependent HAC secretome modulates the activity of these pathways, we used stable luciferase reporter cell lines. Canonical TGF-β signaling activity was measured using the SMAD Binding Element (SBE) reporter 39. Additionally, for non-canonical signaling we utilised a NF-κB luciferase reporter cell line40. Stimulation of the reporter cells with freshly prepared BCP-conditioned medium resulted in a significant increase in SBE and NF-κB reporter activity compared to control (Figure 4B). The activity of both reporters increased around 1.2-fold. Taken together, the differential presence of TGF-β signaling-associated proteins in the BCP-dependent secretome of OA HACs correlated to induction of TGF-β target gene expression and increased activity of the SBE and NF-κB luciferase reporters.

**BCP-driven TGF-β signaling modulates pro-inflammatory cytokine expression by OA chondrocytes**

After having observed the BCP-dependent increase in TGF-β signaling, we aimed to elucidate its biological consequence in relation to expression of the inflammatory cytokine IL-6. For this purpose, we interfered with TGF-β signaling at different levels of the signaling cascade. Due to the multifaceted nature of the TGF-β signaling, we used several inhibitors. On the receptor level we used an ALK5 inhibitor. Further downstream in the signaling cascade the TAK-1 inhibitor and a NF-κB inhibitor (Figure 5A.). Inhibition of ALK5 and TAK-1 were effective in significantly reducing BCP induced *IL-6* to control level*,* while inhibition ofNF-κB had no effect(Figure 5B.). In line with gene expression data, the BCP-driven IL-6 protein secretion could be inhibited by the same set of inhibitors. Opposing to *IL-6* gene expression levels, the NF-κB inhibitor was effective in reducing secreted IL-6 levels. Taken together, increased BCP-driven TGF-β signaling resulted in increased gene expression and secretion of the pro-inflammatory factor IL-6.

Discussion and conclusion

Investigations of the cellular responses to BCP crystals has contributed to a better understanding of their potential role in OA pathology. However, the complexity of the chondrocyte secretomes in response to these CaC was unknown. Therefore, we aimed to characterize the BCP-crystal driven changes in the OA HAC secretome.

The majority of the BCP-dependent changes that we identified revealed an effect on ECM components and ECM remodeling enzymes. Overall, we observed decreased gene expression of healthy cartilage components and increased gene expression and secretion of matrix degrading enzymes. We observed decreased expression of *ACAN* after BCP stimulation. In concordance with our finding, Ea *et* al showed a decrease in proteoglycan content after *in vivo* injection of OCP crystals, a subtype of BCP crystals10. The decrease in proteoglycans observed *in vivo* might therefore be the result of decreased *ACAN* expression.

Alongside downregulation of healthy cartilage ECM components, we observed an increase in MMP-1 and MMP-3. *In vitro* stimulation of human fibroblasts with CaC resulted in an increased expression of MMP-1 in a RAS/MAPK dependent manner30. Additionally, human tenocytes upregulate MMP-3 in response to BCP crystal stimulation41. Our data further confirm that BCP crystals increase OA related ECM degradation in human OA articular chondrocytes.

Alongside the increase in MMP-1 and MMP-3, we observed no increase in *MMP-13* mRNA following BCP stimulation. This was in sharp contrast with the secreted MMP-13 level, which was significantly increased after BCP crystals stimulation. Previous reports show an increase in *MMP-13* in porcine articular chondrocytes in response to BCP stimulation15. The difference between the gene expression levels and the protein levels in our study could be the result of altered translational mechanisms, such as epigenetics or altered translation mechanisms. Several epigenetic regulatory mechanism have been described for MMP-13, such as non-coding RNA’s and microRNAs 42. Various miRNA’s have been shown to regulate translation of *MMP-13* mRNA, including as miR-27a and miR-148a. A downregulation in regulatory miRNAs as a consequence of BCP crystal stimulation could therefore potentially lead to a more efficient translation of *MMP-13*. Additionally, recent evidence suggest an important role for the ribosome in OA43. Besides the canonical cap-dependent translation, adaptation of the translational machinery under stressful conditions can lead to alternative translation44. This could potentially result in the increased protein levels of MMP-13 without affecting the *MMP-13*.

In addition to the ECM remodeling proteins, a majority of DE proteins in our study were related to TGF-β signaling. By combining the observations from both datasets we found an increase in TGF-β superfamily members and proteins involved in activation of latent TGF-β. TGF-β signaling occurs via a variety pathways such as canonical signaling via SMAD2/3 and non-canonical via TAK-138. Based on our luciferase reporter data, BCP dependent TGF-β signaling mainly occurred via ALK5. The source for this increased activity could be the result of enhanced cleavage of TGF-β or signaling via other family members such as Activin A.

In articular cartilage, TGF-β is constitutively produced as a latent protein complex. To activate TGF-β, it has to be cleaved off Latent TGF-β Binding Protein (LTBP) and this can be done by plasmin57. We found a significant increase in the zymogen of plasmin, plasminogen, and it docking molecule Annexin A2. Annexin A2 allows plasminogen and tissue plasminogen activator (tPA) to bind to the cell surface and facilitates conversion of plasminogen to plasmin45. In addition to plasmin, LTBP cleaving activity has been shown for several proteases, including MMP-1346.

We found a significant increase in both Activin A and its receptor Activin RIIA. Activin A signaling is initiated with binding to two type I receptors (i.e. ALK5) and two type II receptors (i.e. Activin RIIA). Cartilage injury, leads to expression of Activin A in an FGF-2 dependent manner47. FGF-2 can released from the pericellular matrix by either injury or loading of articular cartilage48. This could potentially also be the result of BCP crystals stimulation that is considered microcrystalline stress. BCP induced release of FGF-2, as detected in the antibody array, could therefore potentially initiate expression of Activin A with the consequence of increased TGF-β signaling.

Besides the role in cartilage homeostasis, endogenous TGF-β has been linked to the induction of inflammation and fibrosis49. A direct stimulatory effect on *IL-6* has been shown in primary chondrocytes with expression increasing after 4 hours of stimulation 50. We found a significant decrease in BCP dependent expression and secretion of IL-6 in the presence of TGF-β signaling inhibitors. IL-6 was shown to aggravate mineralization in murine chondrocytes17. The mechanism was shown to be modulation of multiple phosphate transporters. We identified a TGF-β driven increase in IL-6 after BCP stimulation, which may lead to a vicious cycle that stimulates mineralisation.

In conclusion, we conducted a dual proteomic analysis in order to characterize the BCP crystal response of OA HACs. We revealed a key role for TGF-β signaling in the BCP crystal response that drives IL-6 expression and secretion. Although a large portion of the DE proteins in our dataset are involved in TGF-β signaling, additional factors that were identified could also be important for the BCP crystal response in OA HACs and require more in-depth investigation. The high prevalence of BCP crystals in OA creates the need for a better understanding of the biological consequences. Our study provides a broader perspective in the biological changes of the OA HAC secretome, thereby elucidating mechanisms that ultimately contribute to a better understanding and potentially to the development of therapeutic strategies.

Figure legends

***Figure 1. BCP crystal stimulation results in increased pro-inflammatory factors and changes the gene expression profile towards a catabolic status in human articular OA chondrocytes.*****A)** Gene expression and protein secretion of pro-inflammatory factor IL-6 in as a result of 24- and 48-hour exposure to 50µg/ml BCP crystals (n=3) **B)** Human articular OA chondrocyte gene expression levels of extracellular matrix components *COL2A1* and *ACAN* were evaluated by RT-qPCR after 24- and 48-hour exposure to 50µg/ml BCP crystals (n=3). **D)** Gene expression levels of matrix degrading enzymes *MMP-1* and *MMP-3* were evaluated by RT-qPCR in human articular OA chondrocytes exposed to 50µg/ml BCP crystals for either 24 or 48 hours. Data are presented as dots reflecting individual values ± 95% CI. Statistical significance was determined with unpaired T-test. \* P<0.05.

**Figure 2. BCP crystals alters the chondrocyte secretome towards a more catabolic profile as determined by LC/MS. A)** Secreted IL-6 levels were determined for the 14 individual patients by sandwich ELISA after 48 hours of exposure to 50µg/ml BCP crystals (n=2 for each patient). **B)** Volcano plot shows depicts differentially expressed proteins in response to BCP crystal stimulation. The non-axial lines represent ±1.5 FC difference and a p value of 0.05. Statistical significance was determined with ANOVA. **C)** Overview of proteins differentially expressed in the LC-MS/MS dataset. **D)** Validation of the mass-spectrometry data by sandwich ELISA determined for MMP-1 and TIMP-1. Change in secreted protein level was determined for each donor (n=2). Data are represented as dots for the individual donors, statistical significance for secreted IL-6 (A) and MMP-1 (E) was determined with an unpaired T-test. \* P<0.05

***Figure 3. Cytokine array of pooled conditioned-medium supports complexity of the BCP crystal response with enriched proteins predominantly belonging to TGF-β superfamily and FGF family.***

**A)** A pool of conditioned media was created for BCP and control OA HACs (n=14 for each pool). Conditioned media were analysed with an antibody array for soluble proteins. Differentially expressed proteins as a result of BCP crystal stimulation are depicted in a volcano plot. The non-axial lines represent ± 2 FC and a p value of 0.05. Upregulated proteins are noted in green. Statistical significance was determined with an unpaired Student’s T-test. **B)** Overview of upregulated protein families in response to BCP crystals. **C)** Validation of VEGF by sandwich ELISA for the 14 individual donors (n=2 for each donor). Statistical significance was calculated with a paired T-test. Data is represented as dots for the individual donors, mean ± 95% CI **D)** Validation of Activin A, MMP-1 and TIMP-1 in the conditioned medium pool (n=3 technical replicates) Statistical significance was determined with unpaired T-test. **E)** Venn diagram which shows he overlap of identified proteins in both analysis methods, an overlap of 14 proteins was observed between both datasets. **F)** Venn diagram depicting the overlap between the differentially expressed genes between both datasets, revealing two mutual proteins MMP-1 and TIMP-1. Data is represented as dot for each replicate, mean ± 95% CI. \* P<0.05

***Figure 4. BCP crystal stimulation of human OA articular chondrocytes activates TGF-β signaling.***

**A)** Gene expression levels of TGF-β target genes *PAI-1, SMAD-7* and *ID-1* in a human OA articular chondrocyte pool (n=12) after 48 hours of BCP crystal exposure (50µg/ml) (n=3). **B)** SW1353 cells were transduced using lentivirus containing luciferase reporter genes SBE-RE and NF-κB-RE. After puromycin selection, transduced SW1353 cells were stimulated with control and BCP stimulated conditioned medium originating from a human OA articular chondrocyte pool (n=7) stimulated for 48 hours. Reporter gene activity was determined after 6 hours of stimulation. Data are presented as dots depicting each replicate, mean ± 95%CI. Statistical significance in both panels is determined with unpaired T-test. \*P<0.05.

***Figure 5. BCP- driven TGF-β signaling contributes to increased secretion of pro-inflammatory factors IL-6 in human OA articular chondrocytes.***

**A)** Schematic representation of the suggested pathway which ultimately results in the observed increase BCP-dependent TGF-β signaling. **B)** Gene expression of IL-6 as a result of 48 hour exposure to 50µg/ml BCP crystals in a pool of human OA articular chondrocytes (n=12) (n=3). Involvement of TGF-β related signaling was studied utilizing ALK5i (SB505124; 5µM), Tak-1i ((5Z)-7-Oxozeaenol; 0.5µM) NF-κBi (parthenolide; 10 µM). **C)** Secreted protein levels of IL-6 were determined after BCP crystal stimulation of a pool of human OA articular chondrocytes (n=12) after 48 hours, in presence or absence of inhibitors. Data are presented as dots depicting each replicate, mean ± 95%CI. Statistical significance in all panels is determined with an ordinary one-way ANOVA with Dunnett’s post-hoc test. \*P<0.05.

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