Discovery of calcite as a new pro-inflammatory calcium-containing crystal in human osteoarthritic synovial fluid

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INTRODUCTION: Intra-articular calcium-containing crystals have become inseparable from osteoarthritis (OA) pathobiology. At the time of total knee arthroplasty (TKA), around 60% of the patients show presence of calcium-containing crystals in their OA synovial fluid while almost all cartilage samples contain these crystals. Estimates are that their presence in OA synovial fluid is even higher with the most important limitation being the detection methods. Polarized light microscopy is routinely used for synovial fluid analysis, but accuracy is limited for calcium crystals. Electron Microscopy is more sensitive but laborious and expensive, and therefore not suitable for clinical use or studies with many samples. An emerging technique in determining crystal composition in body fluids is Raman spectroscopy. In the present study, the calcium-containing crystal composition of human OA synovial fluid (OA-SF) was determined using Raman polarized light microscopy. Additionally, the biological consequences of a newly identified intra-articular calcium-containing crystal calcite (a calcium carbonate polymorph) was determined on human OA fibroblast-like synoviocytes (FLS) and human OA articular chondrocytes (HACs).

METHODS: Synovial fluid samples from advanced knee OA cases were collected (METC permit 2017-0183) prior to total knee arthroplasty. Samples were analysed with an integrated Raman polarized light microscope (Hybriscan Technologies). Birefringent crystals were localized with polarized light microscopy and then scanned with the integrated Raman spectrometer. For each particle, the Raman spectrum from 0-3600 cm^{-1} was measured for 1 s/pixel with a laser power of 10 mW and a 532 nm laser. Calcite crystals were further investigated for their biological effect in human OA articular chondrocytes and human OA fibroblast-like synoviocytes. Both cell types were isolated from TKA surgical waste material and cultured in DMEM/F12 supplemented with 10% FCS, 1% P/S and 1% NEAA and stimulated with 50 µg/ml calcite crystals for 48 hours. The conditioned medium was used to determine secretion of IL-6, CXCL8 and PGE_2 with use of ELISA, and RNA was isolated to perform RT-qPCR. Gene expression levels were normalized to the reference gene *PPIA*. Statistical significance was determined with either a paired Student's T-Test or Wilcoxon signed-rank test based on normal distribution of data. Data are presented as mean ± SEM. Statistical significance is considered with a P. value < 0.05.

RESULTS SECTION: In our cohort of 36 OA-SF samples, all synovial fluids showed a variety of calcium-containing crystals. In agreement with previous studies, samples contained calcium pyrophosphate and basic calcium phosphate crystals. Presence of both crystal types together was never observed in the same sample. We also detected calcium-containing crystal species that have not been reported previously in relation to OA. The majority of OA-SFs (94.4%) showed presence of calcite (calcium carbonate) crystals. Additionally, 27% of the OA-SFs showed presence of dolomite (calcium magnesium carbonate). In 25% of the OA-SF samples both calcite and dolomite were simultaneously detected. Raman spectra of the detected crystals are shown in Figure 1A. To determine the biological relevance of these crystals, we investigated the response of human OA FLS and OA HACs to calcite crystals. OA FLSs exposed to calcite crystals for 48 hours did not change gene expression levels of the pro-inflammatory factors *IL-6*, *CXCL8* and *COX-2*. However, secreted levels of CXCL8 and PGE_2 were significantly increased, while IL-6 showed a trend towards increased secretion (Figure 1B). Moreover, we determined effects of calcite crystals on synovial extracellular matrix gene expression levels. Expression levels of *COL1A1* and *FN1* were significantly decreased, while *COL3A1* remained unchanged. We observed no alterations in the gene expression levels of matrix degrading enzymes *MMP-1*, *MMP-3* and *ADAMTS5*. The effects of calcite crystals on articular chondrocytes seemed to be more detrimental than on FLS. After 48 hours, HACs significantly increased gene expression of all of the measured pro-inflammatory factors (*IL-6, CXCL8, COX-2*). These observations were partially reflected in the secreted IL-6 protein level, a significant increase in PGE_2 levels and a trend for increased CXCL8 (Figure 1C). Additionally, we observed a calcite-driven decreased expression of *COL2A1*, *ACAN* and *COL1A1* accompanied by an increase of *MMP-1*, *MMP-3* and *MMP-13* gene expression.

DISCUSSION: Intra-articular calcium-containing crystals are gaining attention as pathological players in osteoarthritis, making them a viable drug target (1). In this study, we identified calcite crystals in knee OA-SF. Stimulation of OA FLS and OA HACs with calcite crystals resulted in increased pro-inflammatory molecule secretion and alterations of gene expression of extracellular matrix remodeling enzymes. Future studies should focus on increasing the cohort to better determine the prevalence of calcite crystals in OA synovial fluid and eventually in cartilage. Next to determining the prevalence, determination of the underlying cellular responses would be of interest.

SIGNIFICANCE/CLINICAL RELEVANCE: The identification of calcite as a new crystal type in OA-SF underpins the importance of fully characterizing the pathological crystal landscape in OA. Establishing and understanding the role of these specific crystal species provides novel avenues for pharmacological OA disease-modification and endotyping.

IMAGES AND TABLES:

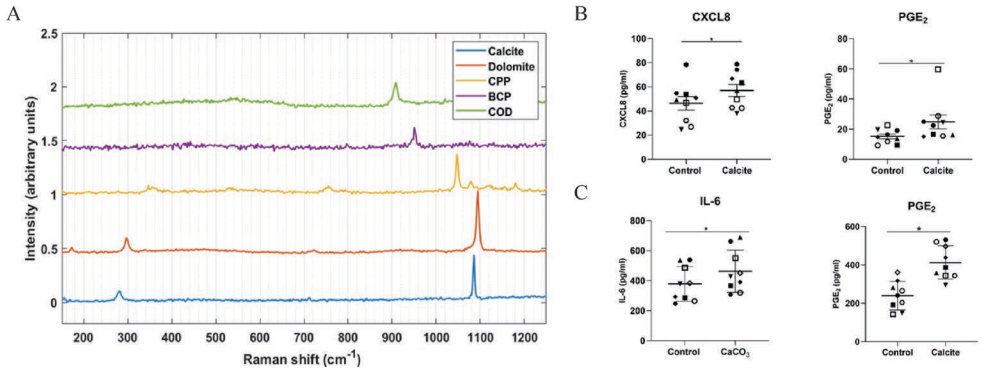

Figure 1. Discovery of calcite crystals in human OA synovial fluid of end-stage OA patients. A) Raman spectrum of calcium-containing crystals in OA synovial fluid of 35 patients. Depicted are the spectra of all detected crystals. B) Secreted protein levels of pro-inflammatory markers by OA FLS of 9 individual donors in response to calcite crystals. C) Secreted proteins levels of pro-inflammatory markers by OA HACs of 9 individual donors in response to calcite crystals. Data in panel B and C are presented as mean ± SEM, n=3 per donor. * P.value <0.05.