INTRODUCTION:
Aggrecanase (ADAMTS-4/-5)-mediated degradation of the aggrecan core protein is a key pathological event in osteoarthritis (OA) that generates specific aggrecan fragments with the N-terminus ARG- or AGEG-, which diffuse from the degrading cartilage into the synovial fluid. Theoretically, these fragments could serve as indicators of cartilage turnover. The goal of the current work was to apply a novel immunoaffinity LC/MS/MS assay, recently developed at Pfizer (Dufield et al, in preparation) in biological fluids of different species.

METHODS:
Assay - A high-throughput immunoaffinity LC/MS/MS assay was used for quantifying ARG- and AGEG-containing aggrecanase-generated aggrecan fragments in synovial fluid (SF) and urine. In brief, the assay utilizes an enzymatic digestion approach to digest one side of the fragment while retaining the biological information from the endogenously generated neoepitope. In this case, chymotrypsin treatment of fluids containing endogenous aggrecan fragments generates the peptides ARG/N/VIL and AGEG/P/SG/SIL, which are then enriched using ARG and AGEG antibody columns. Cartilage explants – Bovine nasal cartilage (BNC) explants were stimulated for 48h with IL-1 ± the aggrecanase-inhibitor, CP669685 (1) and supernatants were collected for measurement of GAGs (with the DMMB assay) and for LC/MS/MS. Rat cartilage degradation model - SF was collected from rats that had received an intra-articular (IA) injection of TNFα in order to trigger rapid cartilage degradation (2), ± intravenous (IV) treatment with CP669685 @ 50 and 300 mg/kg. Human samples - Synovial fluids and urines were analyzed from patients with radiographic knee OA (population 1, n=13; population 2, n=32) and from a control population without knee OA (n=15). Urine levels were normalized for creatinine.

RESULTS:
Validation in IL-1-stimulated BNC: Explants treated with IL-1 for 48 hours showed significant aggrecanase-mediated GAG release, as known (3). A tight correlation exists between GAG levels as determined by DMMB assay and the levels of ARGNSVIL and AGEGPSG/SIL peptides detected in the supernatants by LC/MS/MS (>10 independent expts). To demonstrate that the assay can detect subtle changes in aggrecanase activity, BNC was IL-1 treated in the presence of the aggrecanase inhibitor, CP669685. The inhibitor suppressed GAG release and the generation of both fragments, as detected by LC/MS/MS, in a concentration-dependent manner. The IC50 for the inhibitor as calculated by DMMB assay was 108 nM, whereas the IC50 based on ARGN and AGEG levels was 117 nM and 61 nM, respectively. These findings were reproduced in human cartilage cultures.

ARGN/AGEG fragments in rat synovial fluids - IA administration of TNFα in the rat knee results in a rapid degradation of the articular cartilage, accompanied by the appearance of GAG and ARGN/AGEG epitopes in SF (as detected by Western blot) (2). The LC/MS/MS assay detected ARGNVIL and AGEGSSIL peptides 6h after TNFα injection, peaking at 16h (Fig.1). No ARG/AGEG was detected in the contralateral knee or in knees injected with vehicle control. A dose-dependent suppression of both biomarkers was observed in rats treated IV with the aggrecanase-inhibitor, with the highest dose restoring the levels to baseline.

Human samples - Levels of ARGS and AGEG peptides were significantly higher in SF of patients with radiographic knee OA compared to controls (Fig. 2; two independent knee OA populations). Because a biomarker in a readily accessible fluid would offer a major advantage, levels were measured in the urine of the same patient populations. AGEG was not detectable in urine, but ARGS was detectable, and slightly elevated in OA (Fig. 3); this was reproduced in population 2.

DISCUSSION:
Levels of specific aggrecanase-generated aggrecan neoepitopes can be quantitatively measured in an accurate, sensitive and high-throughput fashion in synovial fluid and in urine. ARGS and AGEG were markedly elevated in the SF of two independent knee OA populations. Also, these neoepitopes correlated with levels of type II collagen fragments, measured as described in (4) (not shown). Although the increase of ARG in OA urine is not dramatic, this assay may be a valuable addition to a previously reported panel of urinary biomarkers such as urinary CTX-II and C2C in OA clinical trials. The results in the rat model indicate that this marker can be modulated in vivo with an aggrecanase-inhibitor, and might thus serve as a proper indicator of target modulation in trials with aggrecanase inhibitors.

REFERENCES:

FIGURES:

![Fig. 1. Levels of AGEGPSG/SIL and ARGNVIL in rat SF after IA injection of TNF (dashed line) or vehicle control (full line)](image1)

![Fig. 2. Levels of AGEGPSG/SIL and ARGNVIL in SF of control and OA patients. * p < 0.05 and ** p < 0.01.](image2)

![Fig. 3. Levels of ARGSVIL in urine of control and OA patients. * p < 0.05. Levels in OA-2 were comparable.](image3)