Conclusions: Pro-inflammatory cytokines are elevated in SF, but not in serum, immediately after knee ACL injury. SF TNF-α level stay elevated over the first 5 years after ACL injury and this high TNF- α concentration may contribute to a later development of OA.

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MULTIPLEX ANALYSIS OF OSTEOARTHRITIC SYNOVIAL FLUID: A COMPARISON OF LUMINEX & MESOSCALE DISCOVERY

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Purpose: Multiplex immunoassay platforms are indispensible for biomarker discovery. They allow high-throughput, multiple parallel analysis of precious small volume samples. The 2 dominant technologies are Luminex (LX) and MesoScale Discovery (MSD). Neither platform is validated for synovial fluid (SF) analysis. The cross-comparison of multiplex formats and comparison against the existing gold standard of ELISA are required to facilitate quality control and guide optimum assay selection for biomarker discovery. Previous comparison studies in culture medium and serum/plasma cannot be extrapolated to the complex biological matrix of SF. **The aim was to make a detailed comparison between LX and MSD platforms during the analysis of real clinical SF samples from end-stage knee OA.**
Methods: SF aliquots from patients with end-stage knee OA (N=31) were analysed for the inflammatory cytokines IL1β, TNFα, IL6 & IL8 using magnetic-bead LX and MSD multiplex assays. All SF samples underwent identical collection, processing, storage and hyaluronidase treatment. Aliquots of an additional SF sample were analysed by each platform after spiking with 2 known concentrations (high & low) of the respective assay calibrator. ELISA for IL6 & TNFα was performed on aliquots from patients previously analysed by the LX (n=8) and MSD (n=11) platforms. The LX and MSD assays were compared for limits of detection (LOD) & quantification (LOQ), spike recovery, and intra-assay precision. Agreement of cytokine measurements between platforms and against ELISA was tested by: weighted Deming Regressions (WDR), concordance correlation coefficient (CCC) for absolute agreement and intraclass correlation coefficient (ICC) for consistency. $P<0.05$ was considered significant.
Results: LOD: The MSD platform had a significantly lower LOD for all 4 analytes ($p<0.01$). IL6 & IL8 were >LOD in all samples on both platforms. IL1β & TNFα were >LOD in significantly more samples on the MSD platform: IL1β 81% vs 3% ($p<0.001$); TNF-α 100% vs 3% ($p<0.001$). **LOQ:** The MSD platform had the lowest LOQ for all 4 analytes, which was significant for all except IL6. Both platforms were able to quantify IL6 & IL8 in >96% of samples. IL1β & TNFα were not quantifiable by the LX assay compared to 29% ($p=0.003$) and 87% ($p<0.001$) by the MSD assay. **Spike-recovery:** Spike recoveries on the MSD platform were acceptable (100±20%) for both concentrations except for the IL6 low-spike (76.6%). IL6 & IL8 had acceptable recoveries for both spikes concentrations on the LX platform. Recoveries at either spike concentrations were below acceptable for IL1β & TNFα. **Intra-assay precision:** The coefficient of variation (CV) for replicate measurements for all analytes was acceptable (<20%) irrespective of platform. There was no significant difference in median CV between platforms. **ELISA validation:** All samples were <LOD on the TNFα ELISA. There was no significant systematic or proportional error for IL6 measurements by the either platform vs ELISA (WDR). There was moderate concordance for MSD vs ELISA (CCC=0.901) and poor concordance for LX vs. ELISA (CCC=0.768) The consistency of measurements for both platforms vs ELISA was excellent (>0.90). **Cross-platform agreement:** There was proportional bias but no systematic bias in IL6 & IL8 measurements between platforms. The concordance of IL6 (CCC=0.62) and IL8 (CCC=0.53) measurements was poor, but the relative agreement was excellent (ICC>0.95).
Conclusions: The MSD platform is better able to detect and quantify low-level analytes (IL1β & TNFα) in OA SF samples than LX. There is poor absolute agreement but excellent relative agreement between platforms. This is most likely due to differences in antibody pairs and kinetics. Cytokine measurements in OA samples are at best semi-quantitative and depend on the platform, assay and manufacturer, thus making comparisons between studies difficult. Biomarker studies should be consistent and explicit regarding assays.

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LONGITUDINAL CHANGE IN SYNOVIAL FLUID AND SERUM LEVELS OF ARGS-AGGRECAN OVER 5 YEARS AFTER ANTERIOR CRUCIATE LIGAMENT INJURY

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Purpose: Aggrecanase cleavage at the 373Glu-374Ala bond in the interglobular domain of aggrecan, releasing N-terminal 374ARGS fragments into synovial fluid (SF-ARGS), is an early key event in arthritis and joint injuries. We have previously shown that SF-ARGS is associated with radiographic progression of knee osteoarthritis (OA) as well as with progression of self-reported pain after meniscectomy. As a part of a randomized controlled study (KANON), initiated to investigate structured rehabilitation of anterior cruciate ligament (ACL) injuries with or without surgical reconstruction, we here report ARGS-aggrecan levels in SF and in serum over 5 years after ACL injury.
Methods: One hundred and twenty-one subjects (26% women, mean age 26 years, standard deviation [SD] 4.9 years) with an ACL rupture to a previously un-injured knee were followed within 3 weeks from trauma (baseline), at 16 weeks, 30 weeks, 1 year, 2 years and 5 years. Blood samples were obtained from all subjects and SF was collected from a subset of subjects (6 subjects had intact series of both SF and serum samples). Twenty-one knee-healthy individuals (38% women, mean age 28 years, SD 9.4 years) were used as reference. Levels of ARGS-aggrecan were measured in all SF and serum samples with an electrochemiluminescence immunoassay using an anti-aggrecan antibody (AHP0022; Invitrogen) for capture, and a monoclonal anti-ARGS (OA-1) for detection. We used Mann-Whitney rank sum test for comparison of ARGS-concentrations at different time points compared to knee-healthy references, and Spearman's rank order correlation for assessment of correlation, based on Shapiro-Wilk test indicating skewed distributions at all time points.
Results: Levels of ARGS-aggrecan in SF and serum correlated (Spearman's rho = 0.245, $p < 0.001$, n = 356) and were overall 10 times higher in SF than in serum: median nM ARGS in SF (25th and 75th percentiles) in ACL injured at all time points combined 1.16 (0.83, 1.94) and 0.13 (0.10, 0.17) in serum.
SF-ARGS levels in the ACL injury group were elevated compared to knee-healthy references at baseline and at 16 weeks after injury, but after 30 weeks or longer, levels were not significantly different from those observed in the reference group (Table 1). ACL injury serum-ARGS levels compared to knee-healthy reference level were significantly elevated at baseline but not at any other time point (Table 1).

Table 1
Cross-sectional ARGS-aggrecan concentrations (nM) in synovial fluid (SF) and serum after ACL injury and in knee-healthy subjects (REF). Values are medians (25th, 75th percentiles) with fold difference of the medians compared to REF. P-values, Mann-Whitney rank sum tests against uninjured references.

	n	SF-ARGS	fold dif.	p-values	n	serum-ARGS	fold dif.	p-values
REF	21	0.85 (0.57, 1.30)	-	-	19	0.112 (0.075, 0.138)	-	-
Baseline	51	6.85 (4.41,17.25)	8.1	<0.001	120	0.158 (0.114, 0.198)	1.4	0.002
16 weeks	50	1.22 (1.00, 1.89)	1.4	0.005	64	0.134 (0.099, 0.168)	1.2	0.073
30 weeks	48	1.10 (0.77, 1.77)	1.3	0.068	63	0.137 (0.107, 0.164)	1.2	0.075
1 year	49	0.95 (0.74, 1.32)	1.1	0.349	63	0.129 (0.105, 0.157)	1.2	0.148
2 years	85	1.07 (0.79, 1.28)	1.3	0.138	118	0.125 (0.094, 0.153)	1.1	0.290
5 years	63	1.01 (0.69, 1.39)	1.2	0.266	115	0.113 (0.091, 0.143)	1.0	0.784