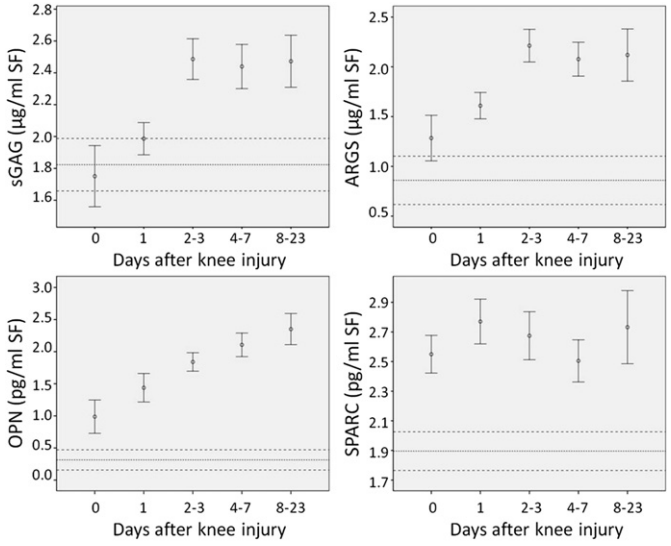
reference subjects. Also, to perform a time dependent analysis and investigate correlations between cartilage and bone biomarkers, and pro-inflammatory cytokines.

Methods: SF was aspirated (0 - 23 days from injury) from the acutely injured knee of 111 individuals (mean age 27 years (13-64), 22% women) with acute joint effusion and blood in the SF (i.e. hemarthrosis). Concentrations of sGAG (sulphated glycosaminoglycan) were measured by Alcian blue precipitation whereas cartilage ARGS, bone biomarker (osteocalcin [OCL], secreted protein acidic and rich in cysteine [SPARC] and osteopontin [OPN]) and pro-inflammatory cytokine (IL-1β, IL-6, IL-8 and TNF-α) concentrations were analyzed using electrochemiluminescence immunoassays. Biomarker concentrations of cartilage, bone and cytokines were compared to SF concentrations from knees of age and sex matched healthy reference subjects and were analyzed with regard to time between injury and aspiration (same day [n=29], 1 day [n=31], 2-3 days [n = 19], 4-7 days [n = 20] and 8-23 days [n = 12]).

Results: SF-concentrations of ARGS ($p<0.001$), SPARC ($p<0.001$), OPN ($p<0.001$), and all cytokines ($p<0.001$), but not sGAG ($p=0.06$) or OCL ($p=0.992$), were significantly higher in injured knees compared to knees of reference subjects. SF concentrations of cartilage markers sGAG and ARGS were significantly elevated in knees aspirated later than 1 day after injury, whereas concentrations of SPARC and OPN were elevated in knees aspirated the same day (day 0) as injury and at all time points thereafter (i.e. day 1-23, fig. 1). The OPN concentrations were higher with increased time between injury and aspiration. Cytokine concentrations were elevated at all time points of aspiration (from day 0-23). Cytokine concentrations were lower with increased time between injury and aspiration at time-points after day 1 (i.e. day 2-23). There were positive correlations between OPN and the cartilage markers (sGAG, $r=0.61$ and ARGS, $r=0.59$), and between SPARC and all measured cytokines ($r>0.33$).

Conclusions: Compared to knees of age and sex matched healthy reference subjects, acutely injured knees with hemarthrosis have elevated SF concentrations of the cartilage biomarker ARGS, bone biomarkers SPARC and OPN and the pro-inflammatory cytokines IL-1β, IL-6, IL-8 and TNF-α. Our results suggest that there could be large variations in these concentrations within the first month of injury and that there may be a relation between OPN and cartilage markers and between SPARC and inflammation. Increased concentrations of SF pro-inflammatory cytokines directly after knee injury may activate proteases leading to degradation of cartilage aggrecan more than 1 day after knee injury.

Fig. 1. sGAG, ARGS, SPARC and OPN concentrations in SF aspirated from the acutely injured knee (hemarthrosis) of individuals at different time points after injury. The data is expressed as the mean log 10 (95% confidence interval) concentrations. The dotted lines represent the mean log10 (95% confidence interval) concentrations in the SF from knees of age and sex matched healthy reference subjects. sGAG: sulphated glycosaminoglycan; SPARC: secreted protein acidic and rich in cysteine; OPN: osteopontin.



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BIOMARKERS OF CARTILAGE DEGRADATION AND SYNTHESIS RELEVANT TO KNEE OSTEOARTHRITIS: RELATIONSHIPS WITH DYNAMIC KNEE JOINT LOAD AND CHANGES FOLLOWING EXERCISE

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Purposes: 1) To examine relationships between biomarkers of articular cartilage degradation and synthesis with dynamic knee joint load in patients with knee osteoarthritis (OA); 2) To compare changes in these biomarkers following 10 weeks of exercise vs. no exercise.

Methods: Seventeen (8 male, 9 female; mean age 66.1, SD 11.3) individuals with radiographically-confirmed medial tibiofemoral OA and varus malalignment participated in this pilot study. All participants underwent a baseline testing session where serum and urine samples were collected, followed by a three-dimensional motion analysis. Motion analysis data from digital cameras and force platforms were combined to calculate the external knee adduction moment (KAM) impulse - a measure of medial compartment knee joint load. Following baseline testing, participants were randomized to either 10 weeks of: 1) physiotherapist-supervised lower limb muscle strengthening exercises, or 2) no exercises (control). Those in the exercise group performed a series of six exercises designed to strengthen the hip abductors, quadriceps, and hamstrings muscles, as well as minimize dynamic varus malalignment. Exercises were performed at home at least four times per week and were partially supervised through five visits (weeks 1, 2, 3, 5, 8) with the study physiotherapist to confirm proper and safe performance and progression of resistance. Identical follow-up testing, including collection of serum and urine samples, was conducted 11 weeks after baseline. Biomarkers assessed included: urinary C-telopeptide of type II collagen (uCTX-II) and urinary type II collagen cleavage neoepitope (uC2C) both normalized for creatinine and indicative of collagen degradation, serum cartilage oligomeric matrix protein (sCOMP) also indicative of cartilage degradation, serum hyaluronic acid (sHA) indicative of synovial inflammation and serum C-propeptide of type II procollagen (sCPII) indicative of collagen synthesis. Ratios of degradation to synthesis biomarker levels were also computed. Biomarker values were log-transformed and linear regression analyses were performed to evaluate the association of each biomarker with the KAM impulse, adjusted for sex, age, and physical activity. Similar linear regression models were computed to assess between-group differences in the change values of each biomarker, adjusting for age and sex.

Results: Nine participants (5 male) were randomized to the exercise group, while the other eight participants (3 male) received no additional treatment during the study. Based on baseline data only, significant positive associations with KAM impulse were only seen for uCTX-II ($p=0.02$) and the ratio of uCTX-II:sCPII ($p<0.01$). Biomarker concentrations (log transformed) at baseline and follow-up for both groups are summarized in Table 1. One participant in the control group was lost to follow-up and did not return for final testing. Compared with the no exercise group, participants in the exercise group reported significant reductions ($p=0.04$) in sCOMP levels following the intervention (non-log transformed reduction of 0.8 U/L vs. 0.9 U/L increase in the non-exercising group). sHA ($p=0.10$) and uCTX-II ($p=0.11$) were only slightly reduced in the exercise group compared to controls.

Conclusions: This research provides initial evidence of the relationship between biomarkers and knee joint load measures in patients with medial tibiofemoral knee OA as well as changes in circulating biomarker concentrations following non-pharmacological treatment. Specifically, it appears that uCTX-II is associated with knee joint load, while sCOMP is amenable to change following exercises specifically designed to offload cartilage.

Mean (sd) natural log-transformed biomarker concentrations for both groups at baseline and follow-up

	Baseline Exercise Group (n=9)	Baseline No Exercise Group (n=8)	Follow-up Exercise Group (n=9)	Follow-up No Exercise Group (n=7)	Adjusted Between-groups Change Difference (95% CI)	p-value
URINARY MARKERS						
uCTX-II (log ng/mmol creatinine)	5.40 (0.81)	5.97 (0.57)	5.32 (0.93)	6.25 (0.68)	-0.33 (-0.71, 0.04)	0.11
uC2C (log ng/mmol creatinine)	2.45 (0.68)	2.46 (0.76)	2.57 (0.81)	2.71 (0.58)	-0.13 (-0.84, 0.58)	0.73
SERUM MARKERS						
sHA (log U/L)	3.47 (0.93)	3.80 (0.96)	3.26 (1.13)	4.21 (0.86)	-0.79 (-1.67, 0.08)	0.10
sCOMP (log U/L)	2.20 (0.21)	2.26 (0.17)	2.11 (0.24)	2.36 (0.13)	-0.16 (-0.30, -0.02)	0.04
sCPII (log U/L)	6.56 (0.19)	6.44 (0.53)	6.50 (0.36)	6.71 (0.40)	-0.34 (-0.93, 0.24)	0.27
RATIOS						
[uCTX-II]:sCPII	-1.16 (0.74)	-0.46 (0.49)	-1.18 (0.97)	-0.46 (0.81)	0.01 (-0.63, 0.66)	0.97
[uC2C]:sCPII	-4.11 (0.69)	-3.98 (1.19)	-3.93 (0.98)	-4.01 (0.73)	0.22 (-0.59, 1.03)	0.61
[sHA]:sCPII	-3.09 (0.99)	-2.64 (0.87)	-3.24 (1.37)	-2.50 (0.77)	-0.45 (-1.43, 0.53)	0.39

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SYNOVIAL FLUID PREPARATION TO IMPROVE IMMUNOASSAY PRECISION FOR BIOMARKER RESEARCH USING MULTIPLEX PLATFORMS

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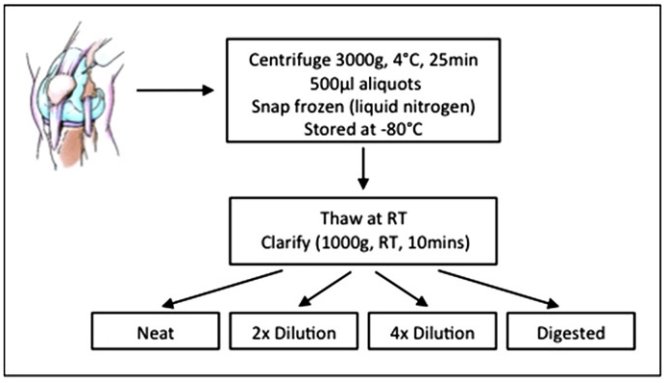
Purpose: Synovial fluid (SF) reflects the biological milieu of the joint and is a potentially important source of biomarkers in osteoarthritis (OA). OA joints usually do not offer enough SF volume for analysis of multiple markers in a single sample by conventional enzyme-linked immunosorbent assays (ELISA). Newer platforms i.e. Luminex and Meso Scale Discovery (MSD) enable multiplex analysis in small (≤50µl) volumes. However, SF analysis is challenging due to its complex matrix and viscous non-Newtonian nature.
The aim of the study was to optimise SF sample preparation for the best assay precision during multiplex analysis by the Luminex and MSD platforms.
Methods: SF was harvested from 16 patients with end-stage knee OA at arthroplasty. All samples underwent identical processing and preparation (Figure 1).



Dilutions were made with the manufacturer's assay diluent. Hyaluronic acid (HA) was digested by treatment with 2mg/ml bovine testicular hyaluronidase (made up in assay diluent) for 1hr at RT. This included an effective 2-fold sample dilution. Experiments (Table 1) were conducted according to the manufacturer's protocol. Samples were analysed in triplicate.

Bead events per target (for Luminex assays only) and the coefficient of variation (CV) of the calculated concentration for sample triplicates were compared for each pre-treatment protocol. Luminex data generated from <35 bead events per target is not acceptable. At least 50 bead events are recommended. Intra-assay precision with a CV <25% is a widely used acceptance criterion. Matched/repeat measure statistical tests were used when possible as each sample was assayed after each pre-treatment. P<0.05 was considered significant.
Results: *Luminex bead counts:* In the polystyrene bead Luminex 10-plex, the proportion of usable samples (≥2 replicates with ≥35 bead events) was significantly greater when samples were digested (McNemar's test, p<0.001 for each target). In the magnetic bead Luminex IL6-plex, all samples were usable with ≥50 bead events in every well for each preparation protocol.
Assay Precision:
Polystyrene bead Luminex 10-plex: Only IL6 and IL8 were above the limit of quantification. Comparison of the overall group mean %CV for all usable samples was significantly lower in digested than neat samples for both IL-6 (p=0.007, Mann-Whitney test) and IL-8 (p=0.004, two-tailed t-test).
Magnetic bead Luminex IL6-plex: Every digested samples had an intra-assay CV <15% (group mean 6.7%). Each sample's intra-assay CV was significantly greater if it was neat or diluted (p=0.0195, repeated measures ANOVA; p< 0.05 for neat, 2-fold & 4-dilution by Newman-Keuls post-hoc comparisons).
*Meso Scale Discovery:*For relatively abundant targets i.e. IL6 & IL8, all but 2 samples had an intra-assay CV <25% for each pre-treatment. There was no significant difference in intra-assay CV for samples under each different pre-treatment (repeat measures ANOVA). For TNF-α, only digestion produced intra-assay CVs <25% for every sample. However, the improved %CV of digested samples did not reach statistical significance (repeat measures ANOVA). ILβ levels were below the linear part of the standard curve at the lower limit of quantification.
Conclusions: The magnetic bead Luminex system maximises bead events per target irrespective of sample preparation.
SF digestion with 2mg/ml hyaluronidase (with 2-fold dilution) gives better intra-assay precision for polystyrene and magnetic bead Luminex assays than dilution alone. MSD assays for abundant targets show good precision irrespective of sample pre-treatment. However, digestion may offer better precision for less expressed targets i.e. TNF-α and is preferable to large dilutions that may take the target below the dynamic range We

Table 1
Experiment Summary

Platform	Platform Info.	Assay Details	Treatment Protocols	Number of Samples
Luminex	Polystyrene bead system	Inflammatory 10-plex (IL1β, IL6, TNFα, IL4, IL8, IL10, GMCSF, IL5, IFNγ & IL2)	Neat Digested	16
Luminex	Magnetic bead system	IL6 single-plex	Neat 2xDilution 4xDilution Digested	7 (of 16)
Meso Scale Discovery	Multi-spot Electrochemiluminescence	Inflammatory 4-plex (IL1β, TNFα, IL6 & IL8)	Neat 2xDilution 4xDilution Digested	7 (of 16) Same as above