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Acute molecular changes in synovial fluid following human knee injury are associated with early clinical outcomes

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Molecular changes in synovial fluid after knee injury

Abstract

Objective: We investigated whether molecules found to be up-regulated within hours of surgical joint destabilisation in the mouse were also elevated in the analogous human setting of acute knee injury, how this molecular response varied between individuals, and whether it related to patient-reported outcomes in the 3 months after injury.

Methods: 7 candidate molecules were analysed in blood and synovial fluid (SF) of 150 participants with recent structural knee injury at baseline (<8 weeks from injury) and in blood at 14 days and 3 months following baseline. $KOOS_4$ was collected at baseline and 3 months. Assays were by MesoScale Discovery™platform or ELISA, and compared with controls.

Results: 6/7 molecules were significantly elevated in human synovial fluid immediately after injury: IL-6, MCP-1, MMP-3, TIMP-1, activin-A and TSG-6. There was low-moderate correlation with blood measurements. 3/6 molecules were significantly associated with baseline $KOOS_4$ (those with higher SFIL-6, TIMP-1 or TSG-6 had lower $KOOS_4$). These 3, MMP-3 and activin-A were all significantly associated with greater improvement in KOOS over 3 months, adjusting for other relevant factors. Of these, IL-6 alone significantly accounted for the molecular contribution to baseline $KOOS_4$, and its difference over 3 months.

Conclusion: Our findings validate relevant human biomarkers of tissue injury identified in a mouse model. Analysis of SF rather than blood more accurately reflects this response. The response is associated with patient-relevant outcomes over this early period, SF IL-6 acting as a single representative marker. Longitudinal outcomes will determine if these molecules are biomarkers of subsequent disease risk.

Molecular changes in synovial fluid after knee injury

Joint injury is a well-established risk factor for osteoarthritis (OA) (1, 2). After meniscal tear requiring surgical intervention, about 50% of individuals will develop OA (3, 4). Other substantial knee injuries, such as intra-articular fractures or anterior cruciate ligament (ACL) ruptures, in isolation or in combination with meniscal tears, also predispose to OA (5-7). However, there are currently no prognostic biomarkers which allow us to reliably predict which individuals with a given knee trauma will develop ongoing symptoms and/or OA. It is clear that surgical ACL reconstruction improves instability symptoms but does not remove the risk of post-traumatic OA. There is a pressing clinical need to identify the early processes in injured joints which lead to subsequent disease, and the factors which influence them. Quantifying an individual's risk of OA may allow us to intervene therapeutically in predisposed individuals.

The setting of joint trauma is arguably an ideal experimental platform to study early or pre-OA states: the initiating stimulus is usually temporally-defined, and validated murine models are in widespread use, allowing investigation of early disease mechanisms (8). Using one such model, by surgical destabilisation of the medial meniscus (DMM), our group has identified the transcriptional events in the first few hours following this procedure, which reproducibly leads within weeks to OA (9). By microarray and subsequent RT-PCR, many genes were found to be up-regulated following joint-destabilising surgery (10). These were mostly inflammatory response genes, including cytokines such as interleukin (IL)-6, chemokines and proteases (including metalloproteinase (MMP)-3 and aggrecanases capable of initiating cartilage degradation), but also several molecules with predicted anti-inflammatory or repair functions, such as activin A, a TGFβ family member, and tumour necrosis factor-stimulated protein-6 (TSG-6). Both the transcriptional response to acute

joint destabilisation and the extent of disease development can be modulated by joint immobilisation, gender or varying genetic strain, suggesting that at least some of this immediate inflammatory gene response is necessary for, or might predict, disease development (10, 11).

Biomarkers for OA have to date focussed primarily on products of matrix degradation, and typically in cohorts of those with established OA. Although some biomarkers show statistically significant associations with severity, progression or therapeutic response (12, 13), this field has been largely disappointing in identifying a qualified biomarker which is of use at the individual level, either in clinical trials or practice (14, 15). Others have documented various elevated inflammatory response molecules or activity of them in synovial fluid after joint injury (16-23). However, with two exceptions most of these studies are cross-sectional and relatively small (N<50), are of a single injury type and lack associated clinical data or the power to examine the effect of clinical variables or outcomes. Few studies simultaneously measure the molecule in synovial fluid and blood, and in control samples (20, 22), and no studies to our knowledge report associations between these measurements and validated prospective patient relevant outcomes. To date 'catabolic' or pro-inflammatory genes of the inflammatory response have typically been examined, rather than those thought to be anti-inflammatory or promoting repair. For example, activin A and TSG-6 have not been investigated in this context.

The Knee Injury Cohort at the Kennedy (KICK) study set out to systematically test whether acute injury response molecules which were upregulated in the mouse joint were also elevated following human knee joint injury, in a specifically designed prospective human cohort. 7 molecules were selected for investigation from our murine studies: IL-1β, IL-6,

MCP-1, MMP-3, TIMP-1, TSG-6 and activin A (10). From a group of ~30 upregulated transcripts, all 7 were highly regulated in the mouse joint, all were predicted to be secreted and therefore quantifiable in human synovial fluid, serum or plasma, and all had assays which were validated by us to reliably measure the analyte in both synovial fluid and blood (Table 1). Here we investigate in this human cohort the presence of this biological response and its association with clinical outcome measures over the initial months following a knee joint injury.

PATIENTS AND METHODS

Ethics. Approval for the KICK study was given by South East London Research Ethics Committee 5 (REC reference 10/H0706/44; NCT0124939). All participants gave written informed consent to participate prior to screening, according to the Declaration of Helsinki.

Participants. Participants were referred for screening by the orthopaedic surgeon investigator (AW) from a population with acute knee injury attending assessment by him at various sites in London, UK. Inclusion criteria were: clinically significant acute knee injury within 8 weeks of recruitment; aged 16-50; knee effusion, evident clinically or by MRI; evidence of ≥1 specified structural injuries on MRI (Table 2). Exclusion criteria were: pre-existing advanced radiographic OA (KL grade 3-4) of the injured (index) knee; inflammatory/ septic arthritis of the index knee; previous or planned knee arthroplasty; active (or treated) systemic inflammatory disease; recent infection; pregnancy; inability to provide blood samples. Relative exclusion criteria included: bony abnormality of the index knee; injury of other body parts or surgery within last 3 months; severe neurological/muscle/hip disease; significant, active co-morbidity; contraindication for MRI.

Clinical outcomes. Knee Injury and Osteoarthritis Outcome Score (KOOS), from which $KOOS_4$, a single composite score can be calculated (an average of 4 of the 5 KOOS subscales including Pain, Symptoms, Sports/Recreation and Quality of Life) (24) and Tegner score (25) were collected at baseline and 3 months (Figure 1A). Musculoskeletal examination including knee effusion grade was documented by the same investigator (FW).

Controls. Normal synovial fluid was from patients undergoing limb amputation for treatment of lower limb tumour, at RNOH Stanmore, London, UK, or transplant donation, at

Charing Cross Hospital, London, UK (REC 09/H0710/60), who had macroscopically normal knee articular cartilage at the time of surgery and no evidence of arthritis or tumour invasion into the joint. Of the controls who had sarcoma, none had received chemotherapy recent to the sample collection.

Plasma or serum was from consenting healthy approximately age- and sex-matched donors (REC 07/H0706/81).

Samples. Biological samples were taken at baseline visit (within 8 weeks of knee injury and prior to any surgical intervention), and subsequently at 14 days and 3 months after baseline (Figure 1A). This included whole blood and, where there was clinical intervention such as arthrocentesis or arthroscopy, synovial fluid. This was collected by needle aspiration from the joint prior to introduction of the arthroscope. All samples were transferred within 2 hours to the laboratory: Whole blood (divided between EDTA and plain tubes for plasma and serum respectively) was centrifuged at 1600G for 15min at 20°C. Synovial fluid was centrifuged for a further 20min at 3000G. Supernatants were stored in cryovials at -80°C in monitored freezers.

Reagents. General laboratory reagents were the best available grade from either Sigma-Aldrich (Dorset, UK) or BDH (Dorset, UK) unless otherwise stated. MSD plates and MSD SULFO-TAG labelled Streptavidin (#R32AD-5) were from Meso Scale Discovery (MSD, Rockville, MD, USA). Activin A Quantikine ELISA was from R&D Systems, MA, USA.

Assays. All assays were carried out as per manufacturers' instructions unless stated otherwise. Each assay underwent structured validation and performance assessment for serum, or plasma, and synovial fluid prior to use (Table 1). ELISA plates were read using

Berthold Mithras LB940 and MSD plates by MSD Sector Imager 2400 and analysed with MSD Discovery Workbench software version 3. For TSG-6, plates were custom-coated (MSD, Rockville, USA) with capture antibody (anti-TSG-6, Clone NG3, (MABT108, Merck Millipore, USA), blocked in 2% BSA/PBS and washed 3 times with PBS/0.05% Tween-20. 25µl/well of standards (250-0.24ng/ml recombinant human TSG-6 (#2104-TS-050; R&D Systems, USA) or samples (in Cusabio sample diluent (MBS926793, MyBioSource, USA) were incubated in duplicate for 2h. 1µl/ml SULFOTAG-labelled streptavidin was added to detection antibody (human TSG-6 biotinylated polyclonal antibody, 0.5µg/ml, (BAF2104; R&D Systems, USA). Plates were washed and 25µl of detection solution added per well for 2h. Plates were washed and incubated with 150µl/well Read Buffer (MSD).

Data storage & statistical analysis. Power calculations to allow detection of a change in outcome of $KOOS_4$, applying MCID of 8, and SD of 15 were carried out at the initiation of the study (24), based on a 2-sided t-test comparing high and low levels of a particular biomarker. 56 individuals in each group (total population of 112) was adequate to give 80% power at a 5% level of significance. Allowing for ~20% drop-out, 150 individuals were recruited.

Comparison of biomarker levels between KICK participants and controls: Normality of each continuous variable was assessed by qq and kernel density plots. For non-parametric variables, differences between 2 groups were compared by Mann-Whitney U test.

Change in biomarker levels over time (baseline, 14 days, 3 months): For comparison of more than 2 groups of normally distributed variables, repeated measures ANOVA was employed.

Association of biomarkers with KOOS: The outcomes were: (i)$KOOS_4$ at baseline; (ii)Absolute change in $KOOS_4$ between baseline and 3-months. Linear regression was employed to model the relationship between biomarker levels and the outcome variable $KOOS_4$, adjusting for 7 pre-defined confounding variables (time from injury to sampling, extent/type of joint injury, synovial fluid blood staining, presence of effusion, gender, age, BMI). Crude unadjusted models describe association of biomarkers with outcome, and adjusted models shown control for 4 significant explanatory variables (time from injury to sampling, extent/type of joint injury, heavy synovial fluid blood staining, age).

All available data were analysed on all participants from relevant visits. Data were stored on a secure online database (System for Collaborative Translational Research, HSS, USA). Analysis was performed in STATA IC 13, StataCorp LP, USA and Graphpad Prism Version 6.03, GraphPad Software Inc, USA.

RESULTS

Study population characteristics. Characteristics for the 150 individuals in the cohort are shown in Table 2. The group was young, and predominantly male: just 29 of the 150 were female. The participants' median Tegner score prior to injury was 10, indicating a high level of physical activity (10 equates to national elite competitive sports). 71% were professional sports players. The majority of participants (143, 95%) had sustained their injury during sporting activity, 76% of these playing either football or rugby. Other modes of injury in included tripping, and skiing or ballet injuries.

Participants were recruited soon after knee injury, with a median time from injury to baseline visit of 17 days (Figure 1A). At baseline, the majority of participants had moderate to severe pain in their index knee >4/10 in severity, and the $KOOS_4$ score showed significant impairment (where 100 is normal, and 0 highly impaired; Table 2). All had evidence of clinical effusion at baseline, or had prior evidence of effusion on MRI. There were a range of structural knee injuries within the cohort; these were categorised via arthroscopic findings (subsequently performed for clinical reasons), supplemented by MRI findings. Injuries tended to be structurally more extensive as the category increases (Table 2). The most common findings were a meniscal tear, an ACL rupture, or both. 145/150 underwent surgical treatment of their injury, 140 of these less than 24 hours after baseline visit (supplementary Table 1 details these surgical interventions). Synovial fluid was available for 136 (91%) of the participants at baseline; some blood staining was seen in the majority of participants (Table 2). Data and sample completeness at other visits is shown in Figure 1A. There was 1 case of septic arthritis at 6 weeks and this participant was withdrawn from subsequent study follow up and from the analysis at this point.

Elevation of candidate molecules in synovial fluid, but not peripheral blood after knee injury. 6 of the 7 analytes, IL-6, MCP-1, activin A, MMP-3, TIMP-1 and TSG-6, were significantly elevated in the synovial fluid of KICK participants, compared with 8 control synovial fluids (Figure 1B). However, values varied widely between individuals, with a substantial number lying within the normal range for some molecules such as IL-6 and TSG-6. IL-1β was below the limit of detection for the assay for all but 1 individual (data not shown).

In contrast, there was no significant elevation of the analytes in the blood of KICK participants at baseline (although some individuals had raised IL-6 or TSG-6 levels) (Figure 1B). Paradoxically, all analytes except for IL-6 were significantly lower in KICK blood compared with the 50 controls.

The longitudinal response to joint injury is detectable in peripheral blood for some molecules, but correlates poorly with synovial fluid. Given the low detectable response in the blood samples at baseline, it was important to assess if there was delayed response in serum or plasma in the 3 months after the baseline visit. IL-6 levels were very low or undetectable; for those with measurable levels at baseline there was a tendency to fall, whereas serum MMP-3 increased compared with baseline levels (Figure 2A). However serum MMP-3 levels were only above the upper limit of normal at 3 months for a few individuals. All individuals who had elevated serum TSG-6 at baseline continued to have significantly elevated serum levels at 3 months. A group within the control population who had relatively higher serum levels of TSG-6 was also evident (Figure 1B). MCP-1, activin A and TIMP-1 levels were low and there was very little detectable change in blood over 3 months (data not shown).

There was moderate to strong correlation between the levels of several synovial fluid analytes in samples at baseline, notably IL-6, TIMP-1 and TSG-6 (Figure 2B). In contrast, there was at best low-moderate correlation between paired plasma or serum and synovial fluid levels for any given analyte, except for CRP (Spearman R 0.88, P<0.0001) (Figure 2B). CRP was not significantly elevated in the synovial fluid or serum of those with joint injury compared with controls, although a few individuals did have elevated serum levels at baseline (Table 2). CRP was substantially lower in the synovial fluid than the serum, likely reflecting generation from a systemic rather than joint source. In view of the lack of any satisfactory surrogate blood marker(s) for the immediate response to joint injury, it was important to focus on the large and detectable response in the synovial fluid.

The variation of the injury response is partly explained by time from, and severity of injury. It was likely that synovial fluid markers would be higher, the closer the sampling was to the time of joint injury. This was true for all analytes: samples collected within 20 days of injury had the highest levels (Figure 3). MMP-3 levels appeared least affected by time from injury to sampling. Untransformed data is shown in Figure 3; on log transformation of marker levels, a linear relationship with time from injury accounted for 15-30% of the variation in markers.

Substantial inter-individual variation in these synovial fluid markers was therefore not accounted for. Log transformed synovial fluid analyte levels were modelled by linear regression with 7 pre-defined explanatory variables: time from injury, type/extent of joint injury, presence of synovial fluid blood staining, presence of large effusion, gender, age, BMI (Table 2; Supplementary data, Table 2). Increasing extent of knee trauma was significantly associated with increases in most analytes. The presence of haemarthrosis (moderate or

severely blood-stained synovial fluid) and age were also significant explanatory variables for some analytes. No significant independent effect on any of the analytes was found for gender, BMI or size of effusion.

The molecular response to injury in synovial fluid is independently associated with $KOOS_4$. Synovial fluid IL-6, TIMP-1 and TSG-6 were each significantly associated with baseline $KOOS_4$ (upper panels, Figure 4A). When all 3 markers were added to a linear regression model of baseline $KOOS_4$, IL-6 alone was significant in representing the molecular response in synovial fluid, alongside blood staining and time from injury (Coeff.-4.0 (-5.92,-2.08); Adj. R squared for model=0.30, contribution of 3 analytes=0.16).

The association of each synovial fluid analyte with change in $KOOS_4$ over 3 months was investigated, adjusting for relevant explanatory variables. In the absence of analyte measurements, only 2% of difference in $KOOS_4$ over the 3 months was explained (injury category was only significant variable). Baseline synovial fluid IL-6, activin A, MMP-3, TIMP-1 and TSG-6 levels were each significantly associated with difference in $KOOS_4$ over 3 months, whereas MCP-1 was not (lower panels, Figure 4A). IL-6 was most strongly associated with difference in $KOOS_4$. When all 5 of these synovial fluid analytes were included in the linear regression model, only synovial fluid IL-6 was independently associated with difference in $KOOS_4$ (Coeff.2.80 (0.89,5.52)).

Those in the highest quartile of synovial fluid IL-6 had significantly lower (worse) $KOOS_4$ at baseline compared with individuals with IL-6 in the lowest quartile, whereas these 2 groups had a similar $KOOS_4$ at 3 months. On average, $KOOS_4$ increased by 10 points more in those with the highest quartile of IL-6 at baseline compared to the lowest over 3 months (Figure 4B). The differences between those with low and high IL-6 held true if subgroups with lower

or higher severity injuries were examined. KOOS domains Pain, Symptoms and Sports & physical activity were all significantly reduced (worse) at baseline in those with high synovial fluid IL-6 (P=0.0002, P=0.0035, P=0.04), to a lesser extent Quality of Life, P=0.07.

Findings were the same if the 4 individuals who had undergone synovial fluid collection 14 days after baseline were included (data shown does not include these individuals). A post-hoc analysis of individuals who reported oral NSAID within 10 days of either their baseline or 3 month visit (49 individuals), and those who did not (101 individuals) suggested that NSAID use had no significant effect on baseline $KOOS_4$ (P=0.90), 3 month $KOOS_4$ (P=0.60) or change in $KOOS_4$ (P=0.595 (-5.83, 10.1), or on level of synovial fluid IL-6 (P=0.45).

DISCUSSION

In a large cohort, nearly all of whom had paired synovial fluid and blood available at baseline, we have shown marked elevation in synovial fluid for 6 out of 7 molecules which were candidates identified from a mouse model of joint destabilisation (10). These findings demonstrate the commonality between the mouse and human response to joint injury and support the utility of such pre-clinical models to investigate pathways in early osteoarthritis.

To our knowledge this is the largest study of its type, systematically examining synovial fluid and blood levels of candidate markers across a variety of knee injuries with longitudinal follow up. Although markers increased with extent of injury, the ubiquitous nature of the response irrespective of the type of injury was striking. Similar increases in IL-6, whether an ACL tear was isolated or associated with meniscal injury, or with low/high energy intra-articular fracture have been reported (22, 26, 27). It may be that the joint injury, rather than its cause, is more important, pointing to an underlying common pathological response to connective tissue injury. The finding that haemarthrosis independently influences biomarkers agrees with previously published work (21). Non-traumatic haemarthrosis has historically been associated with arthritis, and may be pro-inflammatory (28, 29). This is an important consideration as clinical practice moves away from early drainage or washout of joints to more conservative approaches. Our study highlights the importance of having a large enough cohort to adjust for such factors.

It was striking that very little of the synovial fluid response was reflected in the paired blood samples at baseline. Our findings are consistent with recent publications from the KANON cohort, which reported similar levels of sfIL-6 at baseline to us and increased markers of proteolysis in synovial fluid of 121 individuals with ACL rupture, which also correlated poorly

with blood over a 5 year period (22). A further smaller study within 4 weeks of knee injury found that of 7 biomarkers significantly higher in synovial fluid than serum, there was correlation with serum in just 4 (20). In recent years there has been a focus on serum and urine biomarkers for osteoarthritis detection and prognosis (14, 15, 30, 31). Whilst a reliable marker which is detectable in more accessible biological samples remains an important goal, ours and other studies examining both synovial fluid and blood highlight the potential loss in sensitivity if only blood is examined. It may be that in a more chronic setting after injury, the predictive value of some blood-based markers improves (17).

We have identified for the first time TSG-6 and activin A as highly regulated molecules in synovial fluid following human joint injury. Activin A has a role in repair and wound healing (32, 33). We have previously shown it is actively synthesised by cartilage in response to experimental injury, and that it exerts an anti-catabolic effect on IL-1-induced aggrecan degradation *in vitro* (34). TSG-6 forms complexes with hyaluronan and inter-alpha-inhibitor (I-α-I) (35, 36). It reportedly protects against inflammatory arthritis by reducing cartilage and bone turnover (37). Genome-wide linkage analyses associate TSG-6 with OA (38). In OA, those with elevated TSG-6: I-α-I have a higher risk of progression to total joint replacement (39). The magnitude of response of either molecule following joint injury could influence an individual's propensity or otherwise to OA. Findings for the other 4 molecules are in keeping with previous cross-sectional studies in those with joint injury, showing raised synovial fluid levels of IL-6 and MCP-1 (18, 23), MMP-3 (17, 40, 41) and TIMP-1 (16, 19). Maximum levels in synovial fluid of IL-1β after ACL rupture are reported within 24 hours (18). The longer median time to sampling of 17 days may explain our lack of detectable IL-1.

IL-6, TIMP-1 and TSG-6 were each associated with the $KOOS_4$ clinical score at baseline and with its change over a 3 month period. Synovial fluid IL-6 accounted for this independently, making any additional contribution of TIMP-1 or TSG-6 redundant. It is important to interpret these findings cautiously. That higher measurable levels of inflammation, represented here by synovial fluid IL-6, are associated with worse clinical symptoms, such as pain and loss of function is perhaps not surprising. However, it is noteworthy that measurement of this single marker could, independently of other factors, account for as much as 16% of inter-individual clinical variation. Sometimes simple clinical measurements provide the same information as biomarkers, but here measures such as presence of effusion were inferior to synovial fluid IL-6. Although those with high synovial fluid IL-6 were more impaired at baseline, they reached a similar point by 3 months to those with low IL-6, suggesting that the presence of inflammation for any given injury does not appear to be an early adverse prognostic factor (at least following surgical management of the injury). It is possible that a greater inflammatory response may predict a greater associated reparative response by the individual, or simply that there is more room for improvement for these individuals in this timeframe.

The IL-6 response to injury in the joint may be biologically important: it is synthesised by chondrocytes and synoviocytes, has the potential to initiate joint damage, and can sensitise joint nociceptive C-fibres (42). IL-6 is often elevated in established OA and rheumatoid arthritis synovial fluids, and is a therapeutic target for the latter (43). However, genetic deletion of IL-6 has no impact on, or may even worsen, murine OA (44, 45). Alternatively, the IL-6 response may be no more important than the up-regulation of these other molecules, but its measurement most accurately represent the overall molecular response,

or elements of this: synovial fluid IL-6 showed the highest correlation with other analytes (including those associated with repair), the greatest up-regulation after injury and also the greatest variation between individuals. Similar features of the IL-6 response were noted after ACL rupture (22). Interestingly, average pain score after meniscal injury was associated with increased IL-6, MCP-1, MIP-1β or IFNγ in lavaged synovial fluid, but no prospective data or validated patient relevant outcomes were collected (23). In those undergoing partial meniscectomy, histological synovial inflammation was associated with worse pre-operative symptoms, but not with poorer outcomes in the first 2 years after arthroscopy, in support of our findings (46).

Our study has some limitations. We show that examination of synovial fluid appears to have more utility than blood, but were not able to systematically collect interval synovial fluid samples. Only 18 individuals (with ongoing clinical problems) were re-sampled, so analysis of this non-representative subgroup was not included. Systematic longitudinal collection of synovial fluid samples is clearly desirable when possible. The 93% rate of synovial fluid sampling in this cohort was possible because the vast majority of participants underwent planned early surgical interventions. Because of this, it is impossible to distinguish the longitudinal response to injury and that to surgical intervention. It will be important to examine whether the same is found in a conservatively- managed cohort.

Another limitation includes the relatively small number of synovial fluid control samples, and the significantly different age distributions of controls compared with participants. Consideration of 'normal' biomarker levels should be tempered by this. Controls with high levels of sporting activity would have been preferable: the unexpected raised analytes such as MCP-1 in (less active) healthy control blood may reflect high sporting levels being

'immunosuppressive' (47). Interestingly, reduced serum markers following trauma were reported in a young, athletic population, compared with pre-trauma levels (48).

This study has demonstrated a quantifiable cellular response to joint injury, best represented by measurement of synovial fluid IL-6, which varies between individuals and is associated with clinical symptoms measured by $KOOS_4$ in this early period after injury. It will be important to investigate whether any of this early molecular response can predict clinical or radiological outcomes in the years after knee injury.

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Analyte	Assay	Cat #, manufacturer	Intra-assay cv (%)	Inter-assay cv (%)	Normal range serum/ *plasma	Normal range synovial fluid	SF Dilution (fold)
Activin A	Human activin A Quantikine	R&D Systems USA DAC00B	2.6	6.9	128 - 403 pg/ml*	655-5250 pg/ml	50
CRP	Human vascular injury II	K15136C-1 MSD (Rockville, USA)	8.2	15.0	0 - 5000 ng/ml	110 - 2670 ng/ml	1000
IL-1β	Pro-inflammatory 9-plex ultra-sensitive	K15007C-1 MSD (Rockville, USA)	8.0	0.8	< 3 pg/ml*	<3 pg/ml	5
IL-6	Custom multiplex (IL-6 + MCP-1)	K15007C-1 MSD (Rockville, USA)	10.8	15.7	0 - 1.49 pg/ml*	0-19.8 pg/ml	5
MCP-1	Custom multiplex (IL-6 & MCP-1)	K151AYC-1 MSD (Rockville, USA)	6.4	21.7	84 – 499 pg/ml*	55 – 487 pg/ml	5
MMP-3	Human MMP 3-plex ultra-sensitive	K15034C-1 MSD (Rockville, USA)	6.2	19.9	5.3 - 32.0 ng/ml	0 - 231 ng/ml	400
TIMP-1	Human TIMP-1	K151JFC-1 MSD (Rockville, USA)	6.5	8.8	211 - 466 ng/ml	75 - 745 ng/ml	200
TSG-6	Custom human TSG-6 prototype	Prototype, MSD (Rockville, USA)	8.6	18.8	1.3 - 12.3 ng/ml	0 – 3.1 ng/ml	4

Table 1. Characterisation of assays for analytes.

Immunoassays were by commercially-available plate ELISA, or by electrochemiluminescence (MSD, Rockville, USA). The latter included singleplex, multiplex or prototype-printed assays, validated by us. Plasma or serum, depending on assay, or synovial fluid aliquots were brought up to RT and gently vortexed prior to assay. An intra-assay and inter-assay coefficient of variation (c.v.) of <12% and <25% respectively was established for all assays. (N=20 for intra-assay c.v.; minimum N=4 for inter-assay c.v.). The lower and upper limits of quantitation were calculated from standard curves of 3 validation plates. Samples below this lower limit were arbitrarily given half the lower limit of quantitation as their concentration during analyses[22]. Spike recoveries within 80% and 120% were deemed acceptable. Linearity of dilution was confirmed for all 8 assays across the dilution range used. Uninjured control samples for both fluid types were assayed and normal ranges calculated (mean+/-2 SD); N=50 (serum/plasma); N=8 (synovial fluid).

Characteristic (units)	KICK participants Median (range), or mean (SD), Or n (%)*	Control group Median (range), or mean (SD) Or n (%)*
Age (years)	25 (16-50)	32 (21-49)[1]; 48 (41-68)[2]
Number of males/females	121 (86)/29 (14)*	33 (65)/17 (35)*[1]; 4 (50)/4 (50)*[2]
Time from injury at baseline (days)	17 (1-56)	-
Body mass index (kg/m^2)	26 (19-39)	Not available
Tegner score prior to injury	10 (3-10)	-
Tegner score at baseline	2 (1-6)	-
Type of injury		-
Meniscal tear	27 (18)*	
Single ligament rupture only	28 (18)*	
ACL + meniscal tear	61 (41)*	
Severe trauma[3]	34 (23)*	
Clinical effusion at baseline[4]	145 (97)*	-
Synovial fluid, blood staining (n=136)[5]		
None	42 (31)*	6 (75)
Mild	34 (25)*	2 (25)
Moderate	25 (18)*	
Severe	26 (19)*	
Present, ungraded	9 (7)*	
KOOS$_4$, baseline (n=143)	44 (18)	-
KOOS$_4$, 3 months (n=124)	62 (16)	
Serum CRP (ng/ml) at baseline (n=149)	524 (26.8-56700)	485 (43.7-5098)
K-L Grade, baseline (n=150)	0 (0-2)	Not available

Table 2. Participant characteristics.

Key clinical characteristics for the 150 KICK participants and controls are shown. 4 Types of Injury were defined, tending to increasing extent of trauma with increasing category (by arthroscopy where performed, supplemented by MRI).

Control data for [1]blood and [2]synovial fluid samples. Participants vs. controls: there was a significant difference in age, for blood and SF ($P<0.0001$ by Mann Whitney test), but no significant difference in gender ($P>0.05$ for blood and SF, by Fisher's Exact test).

[3]Severe trauma: Combined ligament (>1) rupture, or fracture or dislocation).

[4]Size of effusion was estimated clinically as small (46%), medium (39%) and large (12%). 5 had effusion at time of MRI which had resolved by baseline.

[5]The presence of blood staining in synovial fluid was graded subjectively in normal light conditions using a pre-defined visual grading scale. None: No visible red staining of the SF; Mild: Visible red staining, high level of translucency (finger behind tube visible with low distortion). Moderate: Heavy red staining, low level of translucency (finger behind tube visible with high distortion). Severe: Heavy red staining, opaque (finger behind tube not visible).

Completeness of data for synovial fluid sampling $KOOS_4$ and serum sampling are indicated in parentheses, next to these variables.

FIGURE LEGENDS

Figure 1. Analytes in the synovial fluid and blood of participants with knee injury and healthy controls.

A, Flow chart indicating timings of study visits in KICK over 3 months, and related injury and timing of its clinically indicated surgical treatment (surgical interventions detailed in supplementary Table 1). Completeness of KOOS data (baseline and 3 months) and sample collection is shown. To be eligible for participation, participants had sustained 1 or more of: meniscal tear, cruciate ligament rupture, collateral ligament tear, posterolateral corner injury, traumatic chondral defects, articular or periarticular fracture, patello-femoral or tibio-femoral dislocation within 8 weeks of baseline visit.

B, Synovial fluid (SF) and matched blood samples (blood), either plasma (IL-6, MCP-1, activin A) or serum (all other assays) from KICK participants with knee injury at their baseline visit (Injured) or healthy, age- and sex-matched controls (Control) were assayed for markers of interest. All samples were centrifuged to remove cells. Supernatants were measured in duplicate by electrochemiluminescence or ELISA (Activin A only). Measurements for each of 6 analytes are shown, plotted on a log10 y axis. Bars show median +/-interquartile range. *P<0.05,** P<0.01,***P<0.001,****P<0.0001 by Mann Whitney U test, comparing injured vs. control samples. Endpoints were reached for all SF analytes except for IL-6: 12/136 (8.8%) & TSG-6 8/135 (6%) which were below respective LLOQs.

Figure 2. Change over time of analytes in the blood of KICK participants, and correlation between synovial fluid and blood analytes.

A, Blood samples, either plasma (IL-6) or serum (MMP-3, TSG-6) from individuals with knee injury (KICK) were taken at their baseline (BL) visit (within 8 weeks of injury), and

subsequently at 14 days (14d) and 3 months (3m) after baseline visit and assayed for IL-6 (left), MMP-3 (middle) and TSG-6 (right). Values within an individual over time are connected with a line. Difference over time for each log transformed analyte level was tested by repeated measures ANOVA, ** P<0.01,****P<0.0001, n.s. = not significant, P>0.05. The dotted line represents the calculated upper limit of normal range for each analyte. For IL-6, 120/149 were below LLOQ at baseline, 58/82 at 14d and 104/120 at 3m. For TSG-6, 32/149 were below LLOQ at baseline, 17/53 at 14d, and 19/120 at 3m.

B, Non parametric Spearman Rank tests were performed on untransformed data to determine correlations between synovial fluid (sf), and serum (s) or plasma (p) analytes: all available participant data from the baseline visit was analysed. Strength of correlation by Spearman R coefficient is shown, and P values are given in parentheses. The key shows light grey (R>0.2 and P<0.05), mid-grey and dark grey colour coding is used to highlight increasing strength of correlation.

Figure 3. The biological response to injury in synovial fluid is governed by factors including time from injury.

Measurements of 6 analytes in synovial fluid from the index knee at baseline visit were plotted on a log10 y axis against time from injury to sampling, in days (within 8 weeks of injury). The untransformed data is shown for all analytes. The dotted line represents the calculated upper limit of normal range for each analyte. Spearman R coefficient (R) and P values are shown for each graph.

Figure 4. Synovial fluid analytes including IL-6 are associated with the clinical outcome $KOOS_4$.

Molecular changes in synovial fluid after knee injury

A, Linear regression models of participants' $KOOS_4$ at baseline (upper 2 panels) and of the difference in $KOOS_4$ over 3 months (lower 2 panels) are shown, for each of 6 synovial fluid analytes at baseline. Forest Plots of unadjusted (crude) results are shown on the left, and results adjusted for 4 pre-defined variables (time from injury, injury category, presence of synovial fluid blood staining and age) on the right. The regression coefficient for each analyte and 95% confidence interval (CI) are shown.

B, $KOOS_4$ at baseline (BLKOOS4), at 3 months (mKOOS4) and the difference in $KOOS_4$ over 3 months (KOOSdiff) are shown for KICK participants grouped into quartiles of synovial fluid IL-6 at baseline, group 1 being the lowest and group 4 being the highest quartiles of IL-6. Differences between $KOOS_4$ in the highest quartile of IL-6 group versus the lowest quartile were compared by Mann Whitney U-test, **P<0.01, *P<0.05, n.s. = P>0.05.

Supplementary Table 1. KICK participants undergoing surgical interventions of the index knee between baseline visit and 3 month visit.

The numbers and percentages undergoing different types of surgical intervention for their knee injury within the first 3 months of the study are given, as detailed in Figure 1A. Categories have been selected to be directly related to the injury categories given in Table 2. 144/150 underwent primary surgical treatment of their injury. Of the 6 patients who did not undergo surgery during this period, 2 participants had meniscal tears, 1 had a posterolateral corner sprain, 2 had MCL tears and 1 had an isolated complete ACL rupture (reconstructed subsequently at 8 months). All were managed with appropriate bracing and physiotherapy.

The median time from baseline visit to surgical procedure was <1 day. Of the 5 participants who underwent later surgical intervention within the 3 months, the intervention was within 2 and 6 weeks of baseline.

Of those undergoing single ligament surgery, the majority were ACL reconstructions. Of those undergoing ACL reconstruction, all either hamstring or patellar autografts and some individuals underwent a lateral tenodesis in addition.

4 individuals required a second episode of surgery to the index knee during the first 3 months: 2 required manipulation under anaesthetic only, and 2 required arthroscopic debridement.

Supplementary Table 2. Linear regression of analyte levels with significant explanatory variables.

Linear regression modelling is shown for synovial fluid analytes. Log transformation of synovial fluid analyte levels and time from injury was carried out, to normalise the data for this purpose. The model includes each of 6 synovial fluid analytes, adjusted for 7 pre-defined variables (Age, Log Time from Injury, Blood Staining of synovial fluid, Injury Category/type of injury (see Table 2), BMI and Gender. (Size of effusion at baseline was also included initially but did not contribute significantly for any analyte in addition to these other variables, and substantially weakened the model so is not included here). Regression coefficient (coeff.) and confidence interval (CI) are shown for each variable. The 4 variables which reached significance for any analyte were Age, Log Time from Injury, Blood Staining of

synovial fluid, Injury Category. An adjusted (adj.) R-squared value for each model including the analyte level and all significant explanatory variables is shown.

Molecular changes in synovial fluid after knee injury

Type of surgical intervention	Number (%)
Meniscal surgery (partial resection/repair)	26 (17)
Single ligament repair or reconstruction only	29 (19)
ACL reconstruction and meniscal surgery (partial resection/repair)	57 (38)
Combined (>1) ligament reconstruction	22 (15)
Patellar stabilisation/fracture stabilisation/chondral microfracture only	10 (7)
No surgery	6 (4)*
TOTAL	**150 (100)**

Supplementary Table 1.

Molecular changes in synovial fluid after knee injury

Synovial Fluid Analyte	Regression coeff. (CI) for explanatory variable						Adj. R-squared
	Age	Log Time from injury	Blood staining	Injury category	BMI	Gender	
IL-6	-0.01(-0.05, 0.035)	-1.79 (-2.23, -1.35)	0.39 (-1.11, 0.27)	0.85 (-0.18, 1.88)	-0.006 (-0.083, 0.72)	-0.55 (-1.58, 0.47)	0.45
MCP-1	0.0007 (-0.012, 0.013)	-0.27 (-0.40, -0.15)	0.40 (0.20, 0.60)	0.18 (-0.15, 0.51)	-0.005 (-0.03, 0.17)	0.026 (-0.26, 0.32)	0.31
Activin A	0.005 (-0.003, 0.013)	-0.26 (-0.33, -0.18)	-0.07 (-0.09, 0.054)	0.30 (0.12, 0.48)	0.006 (-0.008, 0.020)	-0.12 (-0.29, 0.063)	0.30
MMP-3	-0.027 (-0.05, -0.001)	0.24 (0.007, 0.49)	0.32 (0.08, 0.71)	1.42 (0.90, 2.08)	0.03 (-0.013, 0.08)	-0.078 (-0.66, 0.50)	0.27
TIMP-1	-0.016 (-0.03, -0.002)	-0.30 (-0.43, -0.16)	0.28 (0.06, 0.50)	0.99 (0.67, 1.31)	-0.009 (-0.033, 0.016)	-0.069 (-0.38, 0.25)	0.43
TSG-6	-0.019 (-0.05, 0.013)	-0.78 (-1.09, -0.48)	0.79 (0.30, 1.28)	1.14 (0.42, 1.85)	0.0072 (-0.47, 0.061)	-0.59 (-1.29, 0.12)	0.38

Supplementary Table 2.

Acute molecular changes in synovial fluid following human knee injury are associated with early clinical outcomes

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Molecular changes in synovial fluid after knee injury

Abstract

Objective: We investigated whether molecules found to be up-regulated within hours of surgical joint destabilisation in the mouse were also elevated in the analogous human setting of acute knee injury, how this molecular response varied between individuals, and whether it related to patient-reported outcomes in the 3 months after injury.

Methods: 7 candidate molecules were analysed in blood and synovial fluid (SF) of 150 participants with recent structural knee injury at baseline (<8 weeks from injury) and in blood at 14 days and 3 months following baseline. $KOOS_4$ was collected at baseline and 3 months. Assays were by MesoScale Discovery™platform or ELISA, and compared with controls.

Results: 6/7 molecules were significantly elevated in human synovial fluid immediately after injury: IL-6, MCP-1, MMP-3, TIMP-1, activin-A and TSG-6. There was low-moderate correlation with blood measurements. 3/6 molecules were significantly associated with baseline $KOOS_4$ (those with higher SFIL-6, TIMP-1 or TSG-6 had lower $KOOS_4$). These 3, MMP-3 and activin-A were all significantly associated with greater improvement in KOOS over 3 months, adjusting for other relevant factors. Of these, IL-6 alone significantly accounted for the molecular contribution to baseline $KOOS_4$, and its difference over 3 months.

Conclusion: Our findings validate relevant human biomarkers of tissue injury identified in a mouse model. Analysis of SF rather than blood more accurately reflects this response. The response is associated with patient-relevant outcomes over this early period, SF IL-6 acting as a single representative marker. Longitudinal outcomes will determine if these molecules are biomarkers of subsequent disease risk.

Molecular changes in synovial fluid after knee injury

Joint injury is a well-established risk factor for osteoarthritis (OA) (1, 2). After meniscal tear requiring surgical intervention, about 50% of individuals will develop OA (3, 4). Other substantial knee injuries, such as intra-articular fractures or anterior cruciate ligament (ACL) ruptures, in isolation or in combination with meniscal tears, also predispose to OA (5-7). However, there are currently no prognostic biomarkers which allow us to reliably predict which individuals with a given knee trauma will develop ongoing symptoms and/or OA. It is clear that surgical ACL reconstruction improves instability symptoms but does not remove the risk of post-traumatic OA. There is a pressing clinical need to identify the early processes in injured joints which lead to subsequent disease, and the factors which influence them. Quantifying an individual's risk of OA may allow us to intervene therapeutically in predisposed individuals.

The setting of joint trauma is arguably an ideal experimental platform to study early or pre-OA states: the initiating stimulus is usually temporally-defined, and validated murine models are in widespread use, allowing investigation of early disease mechanisms (8). Using one such model, by surgical destabilisation of the medial meniscus (DMM), our group has identified the transcriptional events in the first few hours following this procedure, which reproducibly leads within weeks to OA (9). By microarray and subsequent RT-PCR, many genes were found to be up-regulated following joint-destabilising surgery (10). These were mostly inflammatory response genes, including cytokines such as interleukin (IL)-6, chemokines and proteases (including metalloproteinase (MMP)-3 and aggrecanases capable of initiating cartilage degradation), but also several molecules with predicted anti-inflammatory or repair functions, such as activin A, a TGFβ family member, and tumour necrosis factor-stimulated protein-6 (TSG-6). Both the transcriptional response to acute

joint destabilisation and the extent of disease development can be modulated by joint immobilisation, gender or varying genetic strain, suggesting that at least some of this immediate inflammatory gene response is necessary for, or might predict, disease development (10, 11).

Biomarkers for OA have to date focussed primarily on products of matrix degradation, and typically in cohorts of those with established OA. Although some biomarkers show statistically significant associations with severity, progression or therapeutic response (12, 13), this field has been largely disappointing in identifying a qualified biomarker which is of use at the individual level, either in clinical trials or practice (14, 15). Others have documented various elevated inflammatory response molecules or activity of them in synovial fluid after joint injury (16-23). However, with two exceptions most of these studies are cross-sectional and relatively small (N<50), are of a single injury type and lack associated clinical data or the power to examine the effect of clinical variables or outcomes. Few studies simultaneously measure the molecule in synovial fluid and blood, and in control samples (20, 22), and no studies to our knowledge report associations between these measurements and validated prospective patient relevant outcomes. To date 'catabolic' or pro-inflammatory genes of the inflammatory response have typically been examined, rather than those thought to be anti-inflammatory or promoting repair. For example, activin A and TSG-6 have not been investigated in this context.

The Knee Injury Cohort at the Kennedy (KICK) study set out to systematically test whether acute injury response molecules which were upregulated in the mouse joint were also elevated following human knee joint injury, in a specifically designed prospective human cohort. 7 molecules were selected for investigation from our murine studies: IL-1β, IL-6,

MCP-1, MMP-3, TIMP-1, TSG-6 and activin A (10). From a group of ~30 upregulated transcripts, all 7 were highly regulated in the mouse joint, all were predicted to be secreted and therefore quantifiable in human synovial fluid, serum or plasma, and all had assays which were validated by us to reliably measure the analyte in both synovial fluid and blood (Table 1). Here we investigate in this human cohort the presence of this biological response and its association with clinical outcome measures over the initial months following a knee joint injury.

PATIENTS AND METHODS

Ethics. Approval for the KICK study was given by South East London Research Ethics Committee 5 (REC reference 10/H0706/44; NCT0124939). All participants gave written informed consent to participate prior to screening, according to the Declaration of Helsinki.

Participants. Participants were referred for screening by the orthopaedic surgeon investigator (AW) from a population with acute knee injury attending assessment by him at various sites in London, UK. Inclusion criteria were: clinically significant acute knee injury within 8 weeks of recruitment; aged 16-50; knee effusion, evident clinically or by MRI; evidence of ≥1 specified structural injuries on MRI (Table 2). Exclusion criteria were: pre-existing advanced radiographic OA (KL grade 3-4) of the injured (index) knee; inflammatory/ septic arthritis of the index knee; previous or planned knee arthroplasty; active (or treated) systemic inflammatory disease; recent infection; pregnancy; inability to provide blood samples. Relative exclusion criteria included: bony abnormality of the index knee; injury of other body parts or surgery within last 3 months; severe neurological/muscle/hip disease; significant, active co-morbidity; contraindication for MRI.

Clinical outcomes. Knee Injury and Osteoarthritis Outcome Score (KOOS), from which $KOOS_4$, a single composite score can be calculated (an average of 4 of the 5 KOOS subscales including Pain, Symptoms, Sports/Recreation and Quality of Life) (24) and Tegner score (25) were collected at baseline and 3 months (Figure 1A). Musculoskeletal examination including knee effusion grade was documented by the same investigator (FW).

Controls. Normal synovial fluid was from patients undergoing limb amputation for treatment of lower limb tumour, at RNOH Stanmore, London, UK, or transplant donation, at

Charing Cross Hospital, London, UK (REC 09/H0710/60), who had macroscopically normal knee articular cartilage at the time of surgery and no evidence of arthritis or tumour invasion into the joint. Of the controls who had sarcoma, none had received chemotherapy recent to the sample collection.

Plasma or serum was from consenting healthy approximately age- and sex-matched donors (REC 07/H0706/81).

Samples. Biological samples were taken at baseline visit (within 8 weeks of knee injury and prior to any surgical intervention), and subsequently at 14 days and 3 months after baseline (Figure 1A). This included whole blood and, where there was clinical intervention such as arthrocentesis or arthroscopy, synovial fluid. This was collected by needle aspiration from the joint prior to introduction of the arthroscope. All samples were transferred within 2 hours to the laboratory: Whole blood (divided between EDTA and plain tubes for plasma and serum respectively) was centrifuged at 1600G for 15min at 20°C. Synovial fluid was centrifuged for a further 20min at 3000G. Supernatants were stored in cryovials at -80°C in monitored freezers.

Reagents. General laboratory reagents were the best available grade from either Sigma-Aldrich (Dorset, UK) or BDH (Dorset, UK) unless otherwise stated. MSD plates and MSD SULFO-TAG labelled Streptavidin (#R32AD-5) were from Meso Scale Discovery (MSD, Rockville, MD, USA). Activin A Quantikine ELISA was from R&D Systems, MA, USA.

Assays. All assays were carried out as per manufacturers' instructions unless stated otherwise. Each assay underwent structured validation and performance assessment for serum, or plasma, and synovial fluid prior to use (Table 1). ELISA plates were read using

Berthold Mithras LB940 and MSD plates by MSD Sector Imager 2400 and analysed with MSD Discovery Workbench software version 3. For TSG-6, plates were custom-coated (MSD, Rockville, USA) with capture antibody (anti-TSG-6, Clone NG3, (MABT108, Merck Millipore, USA), blocked in 2% BSA/PBS and washed 3 times with PBS/0.05% Tween-20. 25µl/well of standards (250-0.24ng/ml recombinant human TSG-6 (#2104-TS-050; R&D Systems, USA) or samples (in Cusabio sample diluent (MBS926793, MyBioSource, USA) were incubated in duplicate for 2h. 1µl/ml SULFOTAG-labelled streptavidin was added to detection antibody (human TSG-6 biotinylated polyclonal antibody, 0.5µg/ml, (BAF2104; R&D Systems, USA). Plates were washed and 25µl of detection solution added per well for 2h. Plates were washed and incubated with 150µl/well Read Buffer (MSD).

Data storage & statistical analysis. Power calculations to allow detection of a change in outcome of $KOOS_4$, applying MCID of 8, and SD of 15 were carried out at the initiation of the study (24), based on a 2-sided t-test comparing high and low levels of a particular biomarker. 56 individuals in each group (total population of 112) was adequate to give 80% power at a 5% level of significance. Allowing for ~20% drop-out, 150 individuals were recruited.

Comparison of biomarker levels between KICK participants and controls: Normality of each continuous variable was assessed by qq and kernel density plots. For non-parametric variables, differences between 2 groups were compared by Mann-Whitney U test.

Change in biomarker levels over time (baseline, 14 days, 3 months): For comparison of more than 2 groups of normally distributed variables, repeated measures ANOVA was employed.

Association of biomarkers with KOOS: The outcomes were: (i)$KOOS_4$ at baseline; (ii)Absolute change in $KOOS_4$ between baseline and 3-months. Linear regression was employed to model the relationship between biomarker levels and the outcome variable $KOOS_4$, adjusting for 7 pre-defined confounding variables (time from injury to sampling, extent/type of joint injury, synovial fluid blood staining, presence of effusion, gender, age, BMI). Crude unadjusted models describe association of biomarkers with outcome, and adjusted models shown control for 4 significant explanatory variables (time from injury to sampling, extent/type of joint injury, heavy synovial fluid blood staining, age).

All available data were analysed on all participants from relevant visits. Data were stored on a secure online database (System for Collaborative Translational Research, HSS, USA). Analysis was performed in STATA IC 13, StataCorp LP, USA and Graphpad Prism Version 6.03, GraphPad Software Inc, USA.

RESULTS

Study population characteristics. Characteristics for the 150 individuals in the cohort are shown in Table 2. The group was young, and predominantly male: just 29 of the 150 were female. The participants' median Tegner score prior to injury was 10, indicating a high level of physical activity (10 equates to national elite competitive sports). 71% were professional sports players. The majority of participants (143, 95%) had sustained their injury during sporting activity, 76% of these playing either football or rugby. Other modes of injury in included tripping, and skiing or ballet injuries.

Participants were recruited soon after knee injury, with a median time from injury to baseline visit of 17 days (Figure 1A). At baseline, the majority of participants had moderate to severe pain in their index knee >4/10 in severity, and the $KOOS_4$ score showed significant impairment (where 100 is normal, and 0 highly impaired; Table 2). All had evidence of clinical effusion at baseline, or had prior evidence of effusion on MRI. There were a range of structural knee injuries within the cohort; these were categorised via arthroscopic findings (subsequently performed for clinical reasons), supplemented by MRI findings. Injuries tended to be structurally more extensive as the category increases (Table 2). The most common findings were a meniscal tear, an ACL rupture, or both. 145/150 underwent surgical treatment of their injury, 140~~2~~ of these less than 24 hours after baseline visit (supplementary Table 1 details these surgical interventions). Synovial fluid was available for 136 (91%) of the participants at baseline; some blood staining was seen in the majority of participants (Table 2). Data and sample completeness at other visits is shown in Figure 1A. There was 1 case of septic arthritis at 6 weeks and this participant was withdrawn from subsequent study follow up and from the analysis at this point.

Elevation of candidate molecules in synovial fluid, but not peripheral blood after knee injury. 6 of the 7 analytes, IL-6, MCP-1, activin A, MMP-3, TIMP-1 and TSG-6, were significantly elevated in the synovial fluid of KICK participants, compared with 8 control synovial fluids (Figure 1B). However, values varied widely between individuals, with a substantial number lying within the normal range for some molecules such as IL-6 and TSG-6. IL-1β was below the limit of detection for the assay for all but 1 individual (data not shown).

In contrast, there was no significant elevation of the analytes in the blood of KICK participants at baseline (although some individuals had raised IL-6 or TSG-6 levels) (Figure 1B). Paradoxically, all analytes except for IL-6 were significantly lower in KICK blood compared with the 5030 controls.

The longitudinal response to joint injury is detectable in peripheral blood for some molecules, but correlates poorly with synovial fluid. Given the low detectable response in the blood samples at baseline, it was important to assess if there was delayed response in serum or plasma in the 3 months after the baseline visit. IL-6 levels were very low or undetectable; for those with measurable levels at baseline there was a tendency to fall, whereas serum MMP-3 increased compared with baseline levels (Figure 2A). However serum MMP-3 levels were only above the upper limit of normal at 3 months for a few individuals. All individuals who had elevated serum TSG-6 at baseline continued to have significantly elevated serum levels at 3 months. A group within the control population who had relatively higher serum levels of TSG-6 was also evident (Figure 1B). MCP-1, activin A and TIMP-1 levels were low and there was very little detectable change in blood over 3 months (data not shown).

There was moderate to strong correlation between the levels of several synovial fluid analytes in samples at baseline, notably IL-6, TIMP-1 and TSG-6 (Figure 2B). In contrast, there was at best low-moderate correlation between paired plasma or serum and synovial fluid levels for any given analyte, except for CRP (Spearman R 0.88, P<0.0001) (Figure 2B). CRP was not significantly elevated in the synovial fluid or serum of those with joint injury compared with controls, although a few individuals did have elevated serum levels at baseline (Table 2). CRP was substantially lower in the synovial fluid than the serum, likely reflecting generation from a systemic rather than joint source. In view of the lack of any satisfactory surrogate blood marker(s) for the immediate response to joint injury, it was important to focus on the large and detectable response in the synovial fluid.

The variation of the injury response is partly explained by time from, and severity of injury. It was likely that synovial fluid markers would be higher, the closer the sampling was to the time of joint injury. This was true for all analytes: samples collected within 20 days of injury had the highest levels (Figure 3). MMP-3 levels appeared least affected by time from injury to sampling. Untransformed data is shown in Figure 3; on log transformation of marker levels, a linear relationship with time from injury accounted for 15-30% of the variation in markers.

Substantial inter-individual variation in these synovial fluid markers was therefore not accounted for. Log transformed synovial fluid analyte levels were modelled by linear regression with 7 pre-defined explanatory variables: time from injury, type/extent of joint injury, presence of synovial fluid blood staining, presence of large effusion, gender, age, BMI (Table 2; Supplementary data, Table 2~~1~~). Increasing extent of knee trauma was significantly associated with increases in most analytes. The presence of haemarthrosis (moderate or

severely blood-stained synovial fluid) and age were also significant explanatory variables for some analytes. No significant independent effect on any of the analytes was found for gender, BMI or size of effusion.

The molecular response to injury in synovial fluid is independently associated with $KOOS_4$. Synovial fluid IL-6, TIMP-1 and TSG-6 were each significantly associated with baseline $KOOS_4$ (upper panels, Figure 4A). When all 3 markers were added to a linear regression model of baseline $KOOS_4$, IL-6 alone was significant in representing the molecular response in synovial fluid, alongside blood staining and time from injury (Coeff.-4.0 (-5.92,-2.08); Adj. R squared for model=0.30, contribution of 3 analytes=0.16).

The association of each synovial fluid analyte with change in $KOOS_4$ over 3 months was investigated, adjusting for relevant explanatory variables. In the absence of analyte measurements, only 2% of difference in $KOOS_4$ over the 3 months was explained (injury category was only significant variable). Baseline synovial fluid IL-6, activin A, MMP-3, TIMP-1 and TSG-6 levels were each significantly associated with difference in $KOOS_4$ over 3 months, whereas MCP-1 was not (lower panels, Figure 4A). IL-6 was most strongly associated with difference in $KOOS_4$. When all 5 of these synovial fluid analytes were included in the linear regression model, only synovial fluid IL-6 was independently associated with difference in $KOOS_4$ (Coeff.2.80 (0.89,5.52)).

Those ~~with~~ in the highest quartile of ~~raised~~ synovial fluid IL-6 had significantly lower (worse) $KOOS_4$ at baseline compared with individuals with ~~a low~~ IL-6 in the lowest quartile, whereas these 2 groups had a similar $KOOS_4$ at 3 months. On average, $KOOS_4$ increased by 10 points more in those with the highest quartile of IL-6 at baseline compared to the lowest over 3 months (Figure 4B). The differences between those with low and high IL-6 held true if

subgroups with lower or higher severity injuries were examined. KOOS domains Pain, Symptoms and Sports & physical activity were all significantly reduced (worse) at baseline in those with high synovial fluid IL-6 (P=0.0002, P=0.0035, P=0.04), to a lesser extent Quality of Life, P=0.07.

Findings were the same if the 4 individuals who had undergone synovial fluid collection 14 days after baseline were included (data shown does not include these individuals). A post-hoc analysis of individuals who reported oral NSAID within 10 days of either their baseline or 3 month visit (49 individuals), and those who did not (101 individuals) suggested that NSAID use had no significant effect on baseline $KOOS_4$ (P=0.90), 3 month $KOOS_4$ (P=0.60) or change in $KOOS_4$ (P=0.595 (-5.83, 10.1), or on level of synovial fluid IL-6 (P=0.45).

DISCUSSION

In a large cohort, nearly all of whom had paired synovial fluid and blood available at baseline, we have shown marked elevation in synovial fluid for 6 out of 7 molecules which were candidates identified from a mouse model of joint destabilisation (10). These findings demonstrate the commonality between the mouse and human response to joint injury and support the utility of such pre-clinical models to investigate pathways in early osteoarthritis.

To our knowledge this is the largest study of its type, systematically examining synovial fluid and blood levels of candidate markers across a variety of knee injuries with longitudinal follow up. Although markers increased with extent of injury, the ubiquitous nature of the response irrespective of the type of injury was striking. Similar increases in IL-6, whether an ACL tear was isolated or associated with meniscal injury, or with low/high energy intra-articular fracture have been reported (22, 26, 27). It may be that the joint injury, rather than its cause, is more important, pointing to an underlying common pathological response to connective tissue injury. The finding that haemarthrosis independently influences biomarkers agrees with previously published work (21). Non-traumatic haemarthrosis has historically been associated with arthritis, and may be pro-inflammatory (28, 29). This is an important consideration as clinical practice moves away from early drainage or washout of joints to more conservative approaches. Our study highlights the importance of having a large enough cohort to adjust for such factors.

It was striking that very little of the synovial fluid response was reflected in the paired blood samples at baseline. Our findings are consistent with recent publications from the KANON cohort, which reported similar levels of sfIL-6 at baseline to us and increased markers of proteolysis in synovial fluid of 121 individuals with ACL rupture, which also correlated poorly

with blood over a 5 year period (22). A further smaller study within 4 weeks of knee injury found that of 7 biomarkers significantly higher in synovial fluid than serum, there was correlation with serum in just 4 (20). In recent years there has been a focus on serum and urine biomarkers for osteoarthritis detection and prognosis (14, 15, 30, 31). Whilst a reliable marker which is detectable in more accessible biological samples remains an important goal, ours and other studies examining both synovial fluid and blood highlight the potential loss in sensitivity if only blood is examined. It may be that in a more chronic setting after injury, the predictive value of some blood-based markers improves (17).

We have identified for the first time TSG-6 and activin A as highly regulated molecules in synovial fluid following human joint injury. Activin A has a role in repair and~~, being critical to skin~~ wound healing (32, 33). We have previously shown it is actively synthesised by cartilage in response to experimental injury, and that it exerts an anti-catabolic effect on IL-1-induced aggrecan degradation *in vitro* (34). TSG-6 forms complexes with hyaluronan and inter-alpha-inhibitor (I-α-I) (35, 36). It reportedly protects against inflammatory arthritis by reducing cartilage and bone turnover (37). Genome-wide linkage analyses associate TSG-6 with OA (38). In OA, those with elevated TSG-6: I-α-I have a higher risk of progression to total joint replacement (39). The magnitude of response of either molecule following joint injury could influence an individual's propensity or otherwise to OA. Findings for the other 4 molecules are in keeping with previous cross-sectional studies in those with joint injury, showing raised synovial fluid levels of IL-6 and MCP-1 (18, 23), MMP-3 (17, 40, 41) and TIMP-1 (16, 19). Maximum levels in synovial fluid of IL-1β after ACL rupture are reported within 24 hours (18). The longer median time to sampling of 17 days may explain our lack of detectable IL-1.

IL-6, TIMP-1 and TSG-6 were each associated with the $KOOS_4$ clinical score at baseline and with its change over a 3 month period. Synovial fluid IL-6 accounted for this independently, making any additional contribution of TIMP-1 or TSG-6 redundant. It is important to interpret these findings cautiously. That higher measurable levels of inflammation, represented here by synovial fluid IL-6, are associated with worse clinical symptoms, such as pain and loss of function is perhaps not surprising. However, it is noteworthy that measurement of this single marker could, independently of other factors, account for as much as 16% of inter-individual clinical variation. Sometimes simple clinical measurements provide the same information as biomarkers, but here measures such as presence of effusion were inferior to synovial fluid IL-6. Although those with high synovial fluid IL-6 were more impaired at baseline, they reached a similar point by 3 months to those with low IL-6, suggesting that the presence of inflammation for any given injury does not appear to be an early adverse prognostic factor (at least following surgical management of the injury). ~~Alternatively, it~~It is possible that a greater inflammatory response may predict a greater associated reparative response by the individual, or simply that there is more room for improvement for these individuals in this timeframe.

The IL-6 response to injury in the joint may be biologically important: it is synthesised by chondrocytes and synoviocytes, has the potential to initiate joint damage, and can sensitise joint nociceptive C-fibres ~~in the joint~~ (42). IL-6 is often elevated in ~~the synovial fluid of~~ established OA and rheumatoid arthritis synovial fluids, and is a therapeutic target for the latter (43). However, genetic deletion of IL-6 has no impact on, or may even worsen, murine OA (44, 45). Alternatively, the IL-6 response may be no more important than the up-regulation of these other molecules, but its measurement most accurately represent the

overall molecular response, or elements of this: synovial fluid IL-6 showed the highest correlation with other analytes (including those associated with repair), the greatest up-regulation after injury and also the greatest variation between individuals. Similar features of the IL-6 response were noted after ACL rupture (22). Interestingly, average pain score after meniscal injury was associated with increased IL-6, MCP-1, MIP-1β or IFNγ in lavaged synovial fluid, but no prospective data or validated patient relevant outcomes were collected (23). In ~~one reported pilot study of~~ those undergoing partial meniscectomy, histological synovial inflammation ~~measured histologically~~ was associated with worse pre-operative symptoms, but not with poorer outcomes in the first 2 years after arthroscopy, in support of our findings (46).

Our study has some limitations. We show that examination of synovial fluid appears to have more utility than blood, but were not able to systematically collect interval synovial fluid samples. Only 18 individuals (with ongoing clinical problems) were re-sampled, so analysis of this non-representative subgroup was not included. Systematic longitudinal collection of synovial fluid samples ~~and larger numbers of control synovial fluids~~ is clearly desirable when possible. The 93% rate of synovial fluid sampling in this cohort was possible because the vast majority of participants underwent planned early surgical interventions. Because of this, it is impossible to distinguish the longitudinal response to injury and that to surgical intervention. It will be important to examine whether the same is found in a conservatively-managed cohort.

Another limitation includes the relatively small number of synovial fluid control samples, and the significantly different age distributions of controls compared with participants. ~~There are some differences between the controls and cases for both synovial fluids and~~

~~blood.~~ Consideration of 'normal' biomarker levels ~~of biomarkers should~~needs ~~to~~ be tempered by this. Controls with high levels of sporting activity would have been preferable: the unexpected raised analytes ~~levels~~ such as MCP-1 in (less active) healthy control blood may reflect high sporting levels being 'immunosuppressive' (47). Interestingly, reduced serum markers following trauma were reported in a young, athletic population, compared with pre-trauma levels (48).

This study has demonstrated a quantifiable cellular response to joint injury, best represented by measurement of synovial fluid IL-6, which varies between individuals and is associated with clinical symptoms measured by $KOOS_4$ in this early period after injury. It will be important to investigate whether any of this early molecular response can predict clinical or radiological outcomes in the years after knee injury.

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Molecular changes in synovial fluid after knee injury

Analyte	Assay	Cat #, manufacturer	Intra-assay cv (%)	Inter-assay cv (%)	Normal range serum/ *plasma	Normal range synovial fluid	SF Dilution (fold)
Activin A	Human activin A Quantikine	R&D Systems USA DAC00B	2.6	6.9	128 - 403 pg/ml*	655-5250 pg/ml	50
CRP	Human vascular injury II	K15136C-1 MSD (Rockville, USA)	8.2	15.0	0 - 5000 ng/ml	110 - 2670 ng/ml	1000
IL-1β	Pro-inflammatory 9-plex ultra-sensitive	K15007C-1 MSD (Rockville, USA)	8.0	0.8	< 3 pg/ml*	<3 pg/ml	5
IL-6	Custom multiplex (IL-6 + MCP-1)	K15007C-1 MSD (Rockville, USA)	10.8	15.7	0 - 1.49 pg/ml*	0-19.8 pg/ml	5
MCP-1	Custom multiplex (IL-6 & MCP-1)	K151AYC-1 MSD (Rockville, USA)	6.4	21.7	84 – 499 pg/ml*	55 – 487 pg/ml	5
MMP-3	Human MMP 3-plex ultra-sensitive	K15034C-1 MSD (Rockville, USA)	6.2	19.9	5.3 - 32.0 ng/ml	0 - 231 ng/ml	400
TIMP-1	Human TIMP-1	K151JFC-1 MSD (Rockville, USA)	6.5	8.8	211 - 466 ng/ml	75 - 745 ng/ml	200
TSG-6	Custom human TSG-6 prototype	Prototype, MSD (Rockville, USA)	8.6	18.8	1.3 - 12.3 ng/ml	0 – 3.1 ng/ml	4

Molecular changes in synovial fluid after knee injury

Table 1. Characterisation of assays for analytes.

Immunoassays were by commercially-available plate ELISA, or by electrochemiluminescence (MSD, Rockville, USA). The latter included singleplex, multiplex or prototype-printed assays, validated by us. Plasma or serum, depending on assay, or synovial fluid aliquots were brought up to RT and gently vortexed prior to assay. An intra-assay and inter-assay coefficient of variation (c.v.) of <12% and <25% respectively was established for all assays. (N=20 for intra-assay c.v.; minimum N=4 for inter-assay c.v.). The lower and upper limits of quantitation were calculated from standard curves of 3 validation plates. Samples below this lower limit were arbitrarily given half the lower limit of quantitation as their concentration during analyses[22]. Spike recoveries within 80% and 120% were deemed acceptable. Linearity of dilution was confirmed for all 8 assays across the dilution range used. Uninjured control samples for both fluid types were assayed and normal ranges calculated (mean+/-2 SD); N=50 (serum/plasma); N=8 (synovial fluid).

Molecular changes in synovial fluid after knee injury

Characteristic (units)	KICK participants Median (range), or mean (SD), Or n (%)*	Control group Median (range), or mean (SD) Or n (%)*
Age (years)	25 (16-50)	32 (21-49)[1]; 48 (41-68)[2]
Number of males/females	121 (86)/29 (14)*	33 (65)/17 (35)*[1]; 4 (50)/4 (50)*[2]
Time from injury at baseline (days)	17 (1-56)	-
Body mass index (kg/m^2)	26 (19-39)	Not available
Tegner score prior to injury	10 (3-10)	-
Tegner score at baseline	2 (1-6)	-
Type of injury		-
Meniscal tear	27 (18)*	
Single ligament rupture only	28 (18)*	
ACL + meniscal tear	61 (41)*	
Severe trauma[3]	34 (23)*	
Clinical effusion at baseline[4]	145 (97)*	-
Synovial fluid, blood staining (n=136)[5]		
None	42 (31)*	6 (75)
Mild	34 (25)*	2 (25)
Moderate	25 (18)*	
Severe	26 (19)*	
Present, ungraded	9 (7)*	
KOOS$_4$, baseline (n=143)	44 (18)	-
KOOS$_4$, 3 months (n=124)	62 (16)	
Serum CRP (ng/ml) at baseline (n=149)	524 (26.8-56700)	485 (43.7-5098)
K-L Grade, baseline (n=150)	0 (0-2)	Not available

Molecular changes in synovial fluid after knee injury

Table 2. Participant characteristics.

Key clinical characteristics for the 150 KICK participants and controls are shown. 4 Types of Injury were defined, tending to increasing extent of trauma with increasing category (~~. For screening, these were documented by MRI, but subsequent categorisation was~~ by arthroscop~~y~~ic findings where performed, supplemented by MRI).

Control data for [1]blood and [2]synovial fluid samples. Participants vs. controls: there was a significant difference in age, for blood and SF (P<0.0001 by Mann Whitney test), but no significant difference in gender (P>0.05 for blood and SF, by Fisher's Exact test).

[3]Severe trauma: Combined ligament (>1) rupture, or fracture or dislocation).

[4]Size of effusion was estimated clinically as small (~~in~~ 46%), medium ~~in~~ (39%) and large ~~in~~ (12%) ~~of these individuals~~. ~~The other 5 individuals~~5 had ~~evidence of~~ effusion at time of ~~injury on~~ MRI ~~but this~~which had resolved by baseline.

[5]The presence of blood staining in synovial fluid was graded subjectively in normal light conditions using a pre-defined visual grading scale. None: No visible red staining of the SF; Mild: Visible red staining, ~~but~~ high level of translucency (finger behind tube visible with low ~~level of~~ distortion). Moderate: Heavy red staining, ~~and~~ low level of translucency (finger behind tube visible with high ~~level of~~ distortion). Severe: Heavy red staining, ~~and~~ opaque (finger behind tube not visible).

Completeness of data for synovial fluid sampling $KOOS_4$ and serum sampling are indicated in parentheses, next to these variables.

Molecular changes in synovial fluid after knee injury

FIGURE LEGENDS

Figure 1. Analytes in the synovial fluid and blood of participants with knee injury and healthy controls.

A, Flow chart indicating timings of study visits in KICK over 3 months, and related injury and timing of its clinically indicated surgical treatment (surgical interventions detailed in supplementary Table 1). Completeness of KOOS data (baseline and 3 months) and sample collection is shown. To be eligible for participation, participants had sustained 1 or more of: meniscal tear, cruciate ligament rupture, collateral ligament tear, posterolateral corner injury, traumatic chondral defects, articular or periarticular fracture, patello-femoral or tibio-femoral dislocation within 8 weeks of baseline visit.

B, Synovial fluid (SF) and matched blood samples (blood), either plasma (IL-6, MCP-1, activin A) or serum (all other assays) from KICK participants with knee injury at their baseline visit (Injured) or healthy, age- and sex-matched controls (Control) were assayed for markers of interest. All samples were centrifuged to remove cells. Supernatants were measured in duplicate by electrochemiluminescence or ELISA (Activin A only). Measurements for each of 6 analytes are shown, plotted on a log10 y axis. Bars show median +/-interquartile range. *P<0.05,** P<0.01,***P<0.001,****P<0.0001 by Mann Whitney U test, comparing injured vs. control samples. Endpoints were reached for all SF analytes except for IL-6: 12/136 (8.8%) & TSG-6 8/135 (6%) which were below respective LLOQs.

Figure 2. Change over time of analytes in the blood of KICK participants, and correlation between synovial fluid and blood analytes.

A, Blood samples, either plasma (IL-6) or serum (MMP-3, TSG-6) from individuals with knee injury (KICK) were taken at their baseline (BL) visit (within 8 weeks of injury), and

subsequently at 14 days (14d) and 3 months (3m) after baseline visit and assayed for IL-6 (left), MMP-3 (middle) and TSG-6 (right). Values within an individual over time are connected with a line. Difference over time for each log transformed analyte level was tested by repeated measures ANOVA, ** P<0.01,****P<0.0001, n.s. = not significant, P>0.05. The dotted line represents the calculated upper limit of normal range for each analyte. For IL-6, 120/149 were below LLOQ at baseline, 58/82 at 14d and 104/120 at 3m. For TSG-6, 32/149 were below LLOQ at baseline, 17/53 at 14d, and 19/120 at 3m.

B, Non parametric Spearman Rank tests were performed on untransformed data to determine correlations between synovial fluid (sf), and serum (s) or plasma (p) analytes: all available participant data from the baseline visit was analysed. Strength of correlation by Spearman R coefficient is shown, and P values are given in parentheses. The key shows light grey (R>0.2 and P<0.05), mid-grey and dark grey colour coding is used to highlight increasing strength of correlation.

Figure 3. The biological response to injury in synovial fluid is governed by factors including time from injury.

Measurements of 6 analytes in synovial fluid from the index knee at baseline visit were plotted on a log10 y axis against time from injury to sampling, in days (within 8 weeks of injury). The untransformed data is shown for all analytes. The dotted line represents the calculated upper limit of normal range for each analyte. Spearman R coefficient (R) and P values are shown for each graph.

Figure 4. Synovial fluid analytes including IL-6 are associated with the clinical outcome $KOOS_4$.

Molecular changes in synovial fluid after knee injury

A, Linear regression models of participants' $KOOS_4$ at baseline (upper 2 panels) and of the difference in $KOOS_4$ over 3 months (lower 2 panels) are shown, for each of 6 synovial fluid analytes at baseline. Forest Plots of unadjusted (crude) results are shown on the left, and results adjusted for 4 pre-defined variables (time from injury, injury category, presence of synovial fluid blood staining and age) on the right. The regression coefficient for each analyte and 95% confidence interval (CI) are shown.

B, $KOOS_4$ at baseline (BLKOOS4), at 3 months (mKOOS4) and the difference in $KOOS_4$ over 3 months (KOOSdiff) are shown for KICK participants grouped into quartiles of synovial fluid IL-6 at baseline, group 1 being the lowest and group 4 being the highest quartiles of IL-6. Differences between $KOOS_4$ in the highest quartile of IL-6 group versus the lowest quartile were compared by Mann Whitney U-test, **P<0.01, *P<0.05, n.s. = P>0.05.

Supplementary Table 1. KICK participants undergoing surgical interventions of the index knee between baseline visit and 3 month visit.

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The numbers and percentages undergoing different types of surgical intervention for their knee injury within the first 3 months of the study are given, as detailed in Figure 1A. Categories have been selected to be directly related to the injury categories given in Table 2. 144/150 underwent primary surgical treatment of their injury. Of the 6 patients who did not undergo surgery during this period, 2 participants had meniscal tears, 1 had a posterolateral corner sprain, 2 had MCL tears and 1 had an isolated complete ACL rupture (reconstructed subsequently at 8 months). All were managed with appropriate bracing and physiotherapy.

The median time from baseline visit to surgical procedure was <1 day. Of the 5 participants who underwent later surgical intervention within the 3 months, the intervention was within 2 and 6 weeks of baseline.

Of those undergoing single ligament surgery, the majority were ACL reconstructions. Of those undergoing ACL reconstruction, all either hamstring or patellar autografts and some individuals underwent a lateral tenodesis in addition.

4 individuals required a second episode of surgery to the index knee during the first 3 months: 2 required manipulation under anaesthetic only, and 2 required arthroscopic debridement.

Supplementary Table 21. Linear regression of analyte levels with significant explanatory variables.

Linear regression modelling is shown for synovial fluid analytes. Log transformation of synovial fluid analyte levels and time from injury was carried out, to normalise the data for this purpose. The model includes each of 6 synovial fluid analytes, adjusted for 7 pre-defined variables (Age, Log Time from Injury, Blood Staining of synovial fluid, Injury Category/type of injury (see Table 2), BMI and Gender. (Size of effusion at baseline was also included initially but did not contribute significantly for any analyte in addition to these other variables, and substantially weakened the model so is not included here). Regression coefficient (coeff.) and confidence interval (CI) are shown for each variable. The 4 variables which reached significance for any analyte were Age, Log Time from Injury, Blood Staining of

synovial fluid, Injury Category. An adjusted (adj.) R-squared value for each model including the analyte level and all significant explanatory variables is shown.

Molecular changes in synovial fluid after knee injury

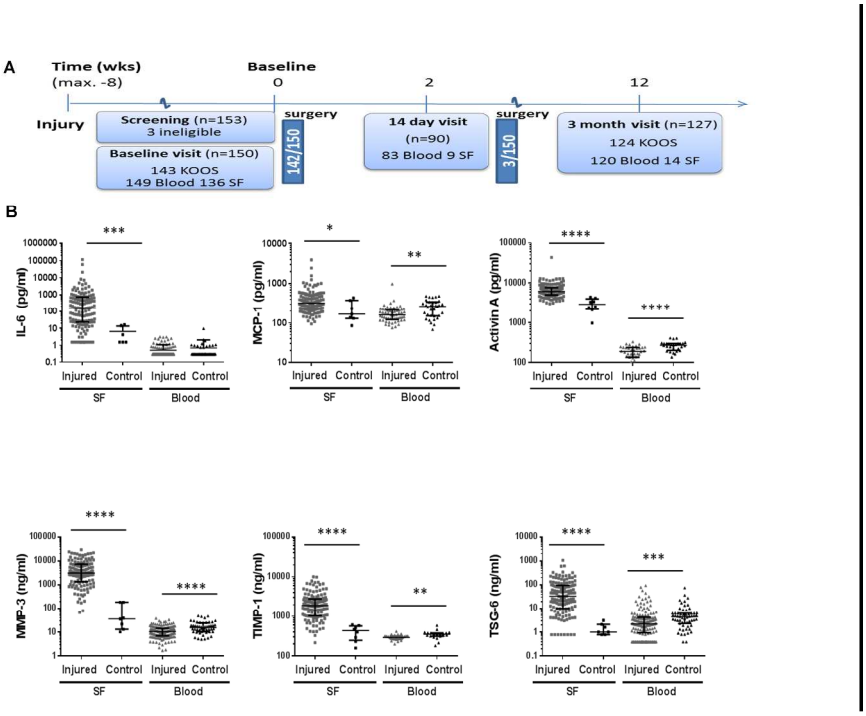
Type of surgical intervention	Number (%)
Meniscal surgery (partial resection/repair)	26 (17)
Single ligament repair or reconstruction only	29 (19)
ACL reconstruction and meniscal surgery (partial resection/repair)	57 (38)
Combined (>1) ligament reconstruction	22 (15)
Patellar stabilisation/fracture stabilisation/chondral microfracture only	10 (7)
No surgery	6 (4)*
TOTAL	**150 (100)**

Supplementary Table 1.

Molecular changes in synovial fluid after knee injury

Synovial Fluid Analyte	Regression coeff. (CI) for explanatory variable						Adj. R-squared
	Age	Log Time from injury	Blood staining	Injury category	BMI	Gender	
IL-6	-0.01(-0.05, 0.035)	-1.79 (-2.23, -1.35)	0.39 (-1.11, 0.27)	0.85 (-0.18, 1.88)	-0.006 (-0.083, 0.72)	-0.55 (-1.58, 0.47)	0.45
MCP-1	0.0007 (-0.012, 0.013)	-0.27 (-0.40, -0.15)	0.40 (0.20, 0.60)	0.18 (-0.15, 0.51)	-0.005 (-0.03, 0.17)	0.026 (-0.26, 0.32)	0.31
Activin A	0.005 (-0.003, 0.013)	-0.26 (-0.33, -0.18)	-0.07 (-0.09, 0.054)	0.30 (0.12, 0.48)	0.006 (-0.008, 0.020)	-0.12 (-0.29, 0.063)	0.30
MMP-3	-0.027 (-0.05, -0.001)	0.24 (0.007, 0.49)	0.32 (0.08, 0.71)	1.42 (0.90, 2.08)	0.03 (-0.013, 0.08)	-0.078 (-0.66, 0.50)	0.27
TIMP-1	-0.016 (-0.03, -0.002)	-0.30 (-0.43, -0.16)	0.28 (0.06, 0.50)	0.99 (0.67, 1.31)	-0.009 (-0.033, 0.016)	-0.069 (-0.38, 0.25)	0.43
TSG-6	-0.019 (-0.05, 0.013)	-0.78 (-1.09, -0.48)	0.79 (0.30, 1.28)	1.14 (0.42, 1.85)	0.0072 (-0.47, 0.061)	-0.59 (-1.29, 0.12)	0.38

Supplementary Table 2~~1~~.



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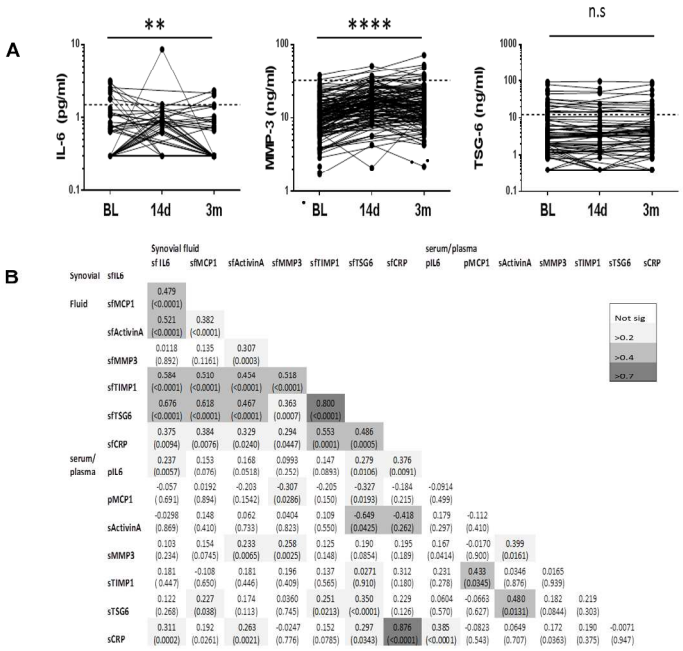

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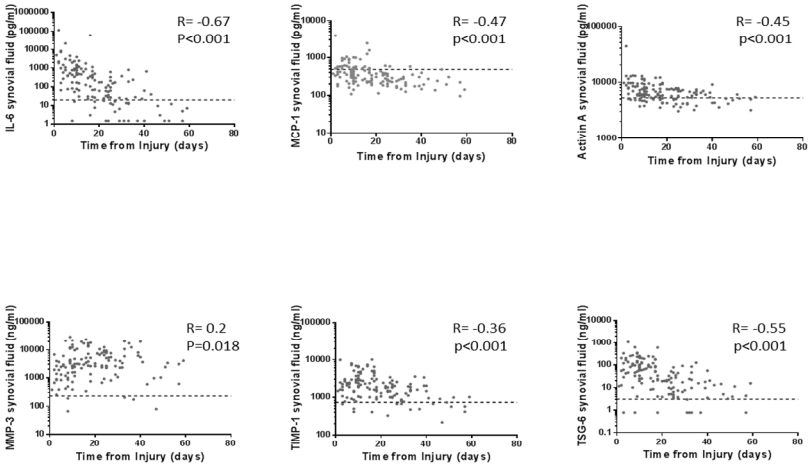

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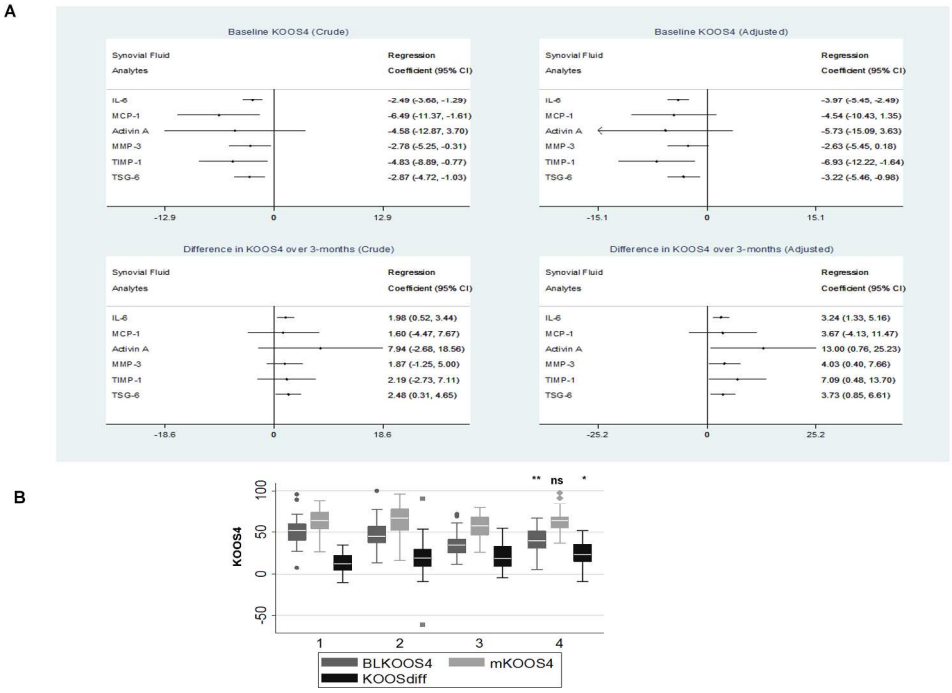

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