Bone & Joint Research

Supplementary Material

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Supplementary Methods

Control patient cohort

The control patient cohort comprised patients with completely asymptomatic knees and normal clinical examination. Individuals were screened to exclude any potential history of knee pathology. Collection of normal control synovial fluid (SF) was attempted from the asymptomatic nonoperative 'good knee' of patients undergoing knee surgery for cruciate ligament or meniscal injuries. Ultrasound-guided needle aspiration of the asymptomatic nonoperative 'good knee' of control patients was attempted under aseptic technique in the operating theatre (after induction of anaesthesia) prior to routine skin preparation and extremity draping of the operative knee.

Despite successful patient recruitment, the ultrasound-guided aspiration of SF from asymptomatic knees in Control patients was unsuccessful and was therefore abandoned after failed attempts in five consecutive patients.

Inflammatory arthritis cohort

Table i. Inflammatory arthritis cohort (n = 18). There were 13 patients with rheumatoid arthritis and five patients with psoriatic arthritis.

Sex	Age, yrs	Arthritis diagnosis	Treatments
F	68	Rheumatoid	Mycophenolate mofetil
F	71	Rheumatoid	Prednisolone, Leflunomide
F	24	Rheumatoid	N/A
M	32	Rheumatoid	Sulphasalazine
F	63	Rheumatoid	Leflunomide
F	72	Rheumatoid	Azothioprine
F	32	Rheumatoid	Methotrexate, Sulphasalazine
M	28	Rheumatoid	N/A
F	85	Rheumatoid	Leflunomide, Adalimumab
F	53	Rheumatoid	Methotrexate, Sulphasalazine, Hydroxychloroquine
F	65	Rheumatoid	Methotrexate, Sulphasalazine, Adalimumab
M	64	Rheumatoid	Methotrexate, Sulphasalazine, Hydroxychloroquine
M	65	Rheumatoid	Sulphasalazine
M	56	Psoriatic	N/A
F	25	Psoriatic	N/A
M	25	Psoriatic	N/A
M	20	Psoriatic	N/A
F	24	Psoriatic	N/A

N/A, not applicable.

Clinical and radiological assessment

Knee osteoarthritis (OA) was diagnosed according to the American College of Rheumatology (ACR) clinical and radiological criteria.[1] Recruited patients' age, sex, and body mass index (BMI) were recorded. Participants were asked to complete the Oxford Knee Score (OKS) questionnaire at preoperative assessment, or on the morning of surgery if not previously obtained.[2] The OKS was also split into ‘functional’ and ‘pain’ components as recently described.[3] Patient age was compared by one-way analysis of variance (ANOVA) with post-hoc Bonferroni multiple comparisons. Between-group comparisons of the OKS, OKS-Pain, and OKS-Function indices were done using the Kruskal-Wallis test with post-hoc Dunn's multiple comparisons.

Patients were assessed with standardized weight-bearing anteroposterior and lateral views, and a patella skyline view of the index knee conducted at preoperative assessment clinic. Radiographs were evaluated using the Kellgren and Lawrence (KL) and Osteoarthritis

Research Society International (OARSI) Atlas grading systems.[4,5] Radiological parameters included the overall (worst) KLG, total OARSI score, and OARSI subscores for joint space narrowing (JSN) and osteophytes (OP). Between-group comparisons of these radiological endpoints used the Kruskal-Wallis test with post-hoc Dunn's multiple comparisons. Patients awaiting arthroscopic surgery, also underwent MRI scanning of the index knee within three months of surgery to exclude OA. MRI scans were assessed qualitatively for the presence of OA features such as chondral thinning, subchondral bone marrow lesions and cysts, osteophytes, and synovitis. There were no clinical or radiological data for the inflammatory cohort, as samples were obtained retrospectively from another study. In all cases, $p < 0.05$ was considered significant.

Wide-spectrum immunoassay analysis

Table ii. Immunoassays. Assays kit names, target analytes, manufacturers, and catalogue numbers are shown. For Luminex assays, *p* = polystyrene bead system, and *m* = magnetic bead system. Samples were acidified, neutralized, and then immediately assayed.

Platform	Assay	Manufacturer	Cat No.
Luminex	*Human Cytokine 25-plex* ^{m} IL-2, IL-12, IL-15, GM-CSF, IL-1Ra, IL-4, IL-10, IL-2R, RANTES, MIP-1α, MIP-1β, MCP-1, IP-10, Eotaxin, and MIG	Life Technologies	LHC0009M
	Bio-Plex Pro Human* *TGF-β 3-plex* ^{m} TGF-β1, TGF-β2, and TGF-β3	Bio-Rad	171-W4001M
	VersaMap Human MMP-13 ^{p}	R&D systems	
MSD	*Human Proinflammatory-4 II* IL-1α, TNF-β, IL-6, and IL-8	MSD	K15025C
	Human MMP 3-plex MMP-1, MMP-3, and MMP-9	MSD	K15034A
	Human TIMP-1	MSD	K151JFC
	Prototype 4-plex BMP-2, BMP-7, LIGHT, and DcR3	MSD	N45ZA-1
ELISA	*Human ADAMTS-4*	CusaBio	CSB-EL0001311HU
	Human COMP	BioVendor	RD194080200

	Human PIIANP	Millipore	EZPIIANP-53K
	Human ARGS neoepitope	GlaxoSmithKlein custom assay[6]	

*Tumour growth factor (TGF)-β required activation prior to detection.

ELISA, enzyme-linked immunosorbent assay; ADAMTS-4, a disintegrin-like and metalloproteinase with thrombospondin motifs-4; ARGS, 374-alanine-arginine-glycine-serine neoepitope of aggrecan; BMP, bone morphogenetic protein; COMP, cartilage oligomeric matrix protein; DcR3, decoy receptor 3; GM-CSF, granulocyte macrophage-colony stimulating factor; IL, interleukin; LIGHT, homologous to Lymphotoxin, exhibits Inducible expression and competes with HSV Glycoprotein D for Herpesvirus entry mediator, a receptor expressed on T cells; MCP, monocyte chemoattractant protein; MIG, monokine induced by gamma interferon; MIP, macrophage inflammatory protein; MMP, matrix metalloproteinase; MSD, MesoScale Discovery; PIIANP, procollagen type IIA N-terminal propeptides; RANTES, Regulated on Activation, Normal T Expressed and Secreted; TIMP, tissue inhibitor of metalloproteinases; TNF, tumour necrosis factor.

Quality control and data preparation

All immunoassay raw data were exported from native software to MasterPlex QT 2010 (Hitachi Solutions, San Francisco, California, USA) to standardize analysis. Background (blank) subtracted signals were used for calibrator points and samples. A five-parameter nonlinear regression model with $1/y^2$ was used for standard curve fitting and concentration calculation.[7] Acceptance criteria for back-calculated concentrations of the standard curve were a relative error within ± 20% of nominal and a coefficient of variation (CV) of ≤ 20% for replicates.[8-10] At least 75% of the calibrator points needed to meet these criteria for the standard curve and assay to be acceptable.

The lower limit of quantification (LOQ) and upper limit of quantification (ULOQ) were defined from the standard curve as the lowest and highest calibrators with back-calculated concentrations ± 20% of nominal and replicate coefficient of variation ≤ 20%. Individual

LOQs and ULOQs for each analyte were recorded from every assay plate that was run during the course of the study. Since the calibrator points of a standard curve are arbitrary discrete concentrations, the average LOQ and ULOQ for an assay were described by the median from the serial plate runs. These average limits were then applied to the analyte concentrations from patient samples analyzed in the study.

The intra-assay precision was described by the CV for measured replicate concentrations. A CV $\leq$ 20% was considered acceptable, unless replicate concentrations were between LOQ and 2LOQ, or 3/4 ULOQ and ULOQ when $\leq$ 25% was satisfactory i.e. at the tails of the standard curve.

Rationale for partial least square discriminant analysis

Partial least square (PLS) modelling assumes that the investigated system or process is influenced by just a few underlying latent variables. Both X- and Y-variables are assumed to be realizations of these underlying latent variables, and are hence not assumed to be independent. Therefore, PLS is philosophically suited for the modelling of biological data. PLS and related methods inherently lend themselves to biomarker discovery where complex datasets have many variables, relatively few patients, and the potential for missing values. Information is extracted by examining all data simultaneously and is robust to correlation between variables and noise in both X and Y variables.[11] PLS methods are frequently used in agriculture, environmental sciences, physical sciences, and other biological fields. Medical disciplines commonly using the approach include oncology, neuroscience, and microbiology, especially when metabolomic and proteomic techniques are employed.

Model diagnostics

The normal probability plot of residuals

This displays the PLS-regression residuals standardized on a double log scale. The standardized residual is the raw residual divided by the residual SD (RSD). This plot allows detection of outliers and assessment of the normality of the residuals. If the residuals are random and normally distributed, the normal probability plot of the residuals has all the points lying on a straight line between -4 and +4 standardized SDs. Experimental runs lying outside the -4 or +4 SDs are outliers.

The permutations plot

This helps to assess the risk that the current model is spurious, i.e. the model just fits the training set well but does not predict the outcome well for new observations. The R^2 and Q^2 values of the original model are compared to those from several models generated by the order of the outcome (Y-observations), which has been randomly permuted, while the predictor (X) matrix has been kept intact. The plot shows, for a selected outcome variable, on the vertical axis the values of R^2 and Q^2 for the original model (far to the right) and of the permuted models further to the left. The horizontal axis shows the correlation between the permuted Y-vectors and the original Y-vector for the selected outcome variable. The original outcome (Y) has the correlation 1.0 with itself, defining the high point on the horizontal axis. The criteria for validity are: 1) all permuted blue Q^2-values to the left are lower than the original points to the right; 2) the blue regression line of the Q^2-points intersects the vertical axis (on the left) at, or below zero; and 3) all green R^2-values to the left are lower than the original point to the right.

Supplementary Results

Clinical and radiological assessments

A summary of the clinical and radiological characteristics of patients with end-stage knee osteoarthritis (esOA), knee injury, and inflammatory arthritis are presented in Supplementary Table iii. The three training cohorts had statistically significantly different ages. Those with knee injuries (approx. 25 years) were the youngest, and the inflammatory cohort (approx. 50 years) were aged approximately halfway between the esOA and injury patients. Men predominated in the esOA and injury cohorts, whereas women predominated in the inflammatory cohort. The median OKS, OKS-Pain, and OKS-Function indices were significantly better (higher) in patients with knee injuries than the esOA cohorts. By definition, patients with knee injury have no radiological features of OA so all radiological scores were significantly higher in the esOA cohort. Features of OA were further excluded in knee injury patients by MRI and intraoperative arthroscopic assessments.

Table iii. Clinical and radiological assessments for study cohorts (training set). No clinical or radiological assessments were available for the inflammatory cohort because samples were obtained from a previous study. Means (and 95% confidence intervals (CIs)) are given for age. Medians (and interquartile ranges (IQRs)) are given for Oxford Knee Score (OKS), OKS Pain, and Function indices; and all radiological parameters. Radiological data shown are: Kellgren & Lawrence grade (KLG); total Osteoarthritis Research Society International (OARSI) score; OARSI joint space narrowing (JSN) subtotal; and OARSI osteophyte (OP) subtotal. One-way analysis of variance (ANOVA) with post-hoc Bonferroni multiple comparisons, or the Kruskall-Wallis test with post-hoc Dunn multiple comparisons, were used as appropriate.

Variable	esOA	Injury	Inflammatory
Patients, n	60	20	18
Sex, m:f	38:22	16:4	7:11
Mean age, yrs (95% CI)	70.0 (67.2 to 72.3)	26.0 (22.9 to 29.1)	48.4 (37.7 to 59.2)*
Median OKS (IQR)	19 (14.25 to 25.5)	40 (35.75 to 42.0)*	N/A
Median OKS-Pain (IQR)	36 (21 to 46)	79 (68.75 to 86.0)*	N/A
Median OKS-Function (IQR)	50 (35 to 64)	90 (85.75 to 94.75)*	N/A
Median KLG (IQR)	3 (3 to 4)	0	N/A
Median OARSI Total (IQR)	11 (7 to14)	0	N/A
Median OARSI JSN (IQR)	3 (2 to 4)	0	N/A
Median OARSI OP (IQR)	7 (3.5 to 9)	0	N/A

*Statistically significant ($p < 0.05$).

esOA, end-stage knee osteoarthritis; N/A, not available.

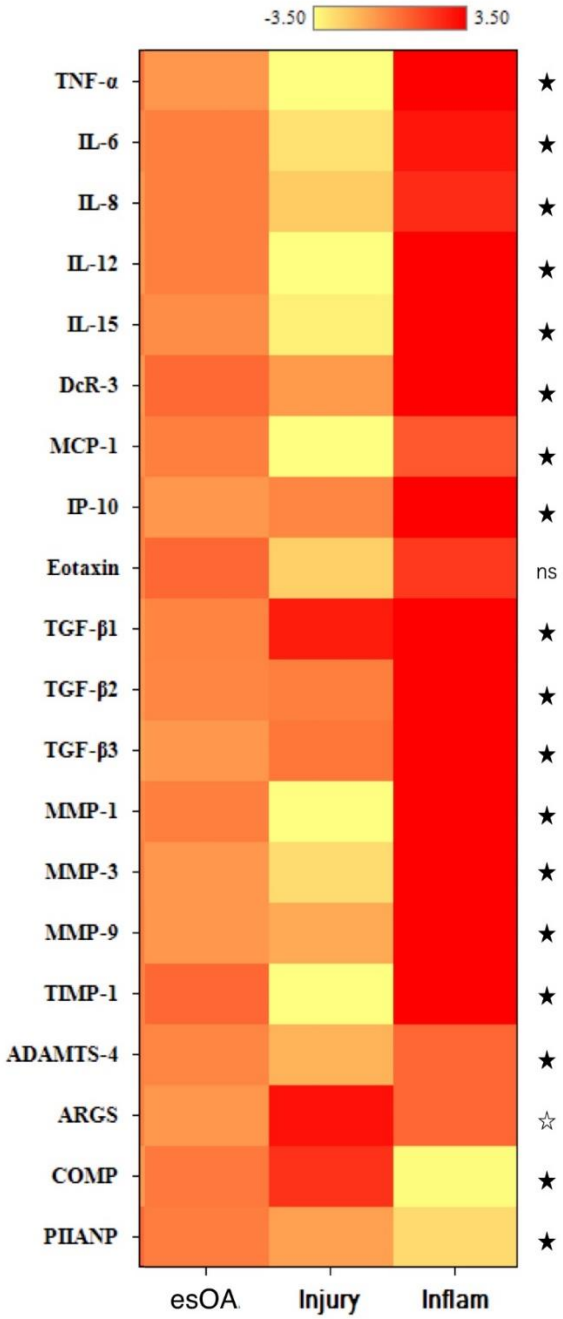

Fig. a. Heat map of standardized median SF analyte concentrations by cohort. Median analyte concentrations for each cohort were natural logarithm transformed and then standardised (robust centre scaling). The Kruskal-Wallis test was used to assess differences across cohorts for raw concentrations of each analyte (non-transformed and non-standardized). A p-value < 0.05 was significant. Stars (★) denote significance after correction for multiple comparisons by Holm's method. Clear stars (☆) denote significance lost after Holm's correction. esOA, end-stage knee OA; Inflam, inflammatory arthritis; Injury, non-OA knee injury.

Non-quantifiable markers

Nine markers (IL-1β, GM-CSF, IL-1RA, IL-2R, RANTES, MIP-1α, MIG, BMP-2, and LIGHT) were sufficiently quantifiable only in inflammatory samples. The final six markers (IL-2, IL-4, IL-10, MIP-1β, MMP-13, and BMP-7) were not quantifiable in sufficient numbers of patients in any cohort. Non-quantifiable markers are presented in Supplementary Table iv.

Table iv. Proportion of synovial fluid (SF) samples above the lower limit of qualification (LOQ) for markers not qualified for quantitative concentration analysis. The proportion of samples above LOQ is given as a percentage for each cohort: end-stage knee OA (esOA), knee injury, and inflammatory arthritis (Inflam). Proportions were compared using chi-squared test (Monte Carlo method) with post-hoc testing by the Marascuilo procedure. A p-value < 0.05 was considered statistically significant. All significant p-values were resilient to adjustment by the Holm step-down method. Significant grouping structure following post-hoc comparisons were A < B < C, {A≈B} < C or A < {B≈C}.

Biomarker	Injury[A] (%)	esOA[B] (%)	Inflam[C] (%)	p-value	Post-hoc grouping
IL-1β	10.5	40	100	< 0.001	A < B < C
IL-2	0	0	6.7	0.150	N/A
GM-CSF	5.3	0	53.3	< 0.001	{A ≈ B} < C
IL-1Ra	0	31.6	100	< 0.001	A < B < C
IL-4	0	1.8	13.3	0.083	-
IL-10	0	0	46.7	< 0.001	{A ≈ B} < C
IL-2R	0	33.3	93.3	< 0.001	A < B < C
RANTES	36.8	24.6	80	< 0.001	{A ≈ B} < C
MIP-1α	0	36.8	86.7	< 0.001	A < B < C
MIP-1β	0	5.3	46.7	< 0.001	{A ≈ B} < C
MIG	0	10.5	60	< 0.001	A < B < C
BMP-2	0	20.7	55.6	0.001	A < B < C
BMP-7	0	0	0	N/A	N/A
MMP-13	5.0	46.6	46.2	0.003	A < {B ≈ C}
LIGHT	0	1.7	83.3	< 0.001	{A ≈ B} < C

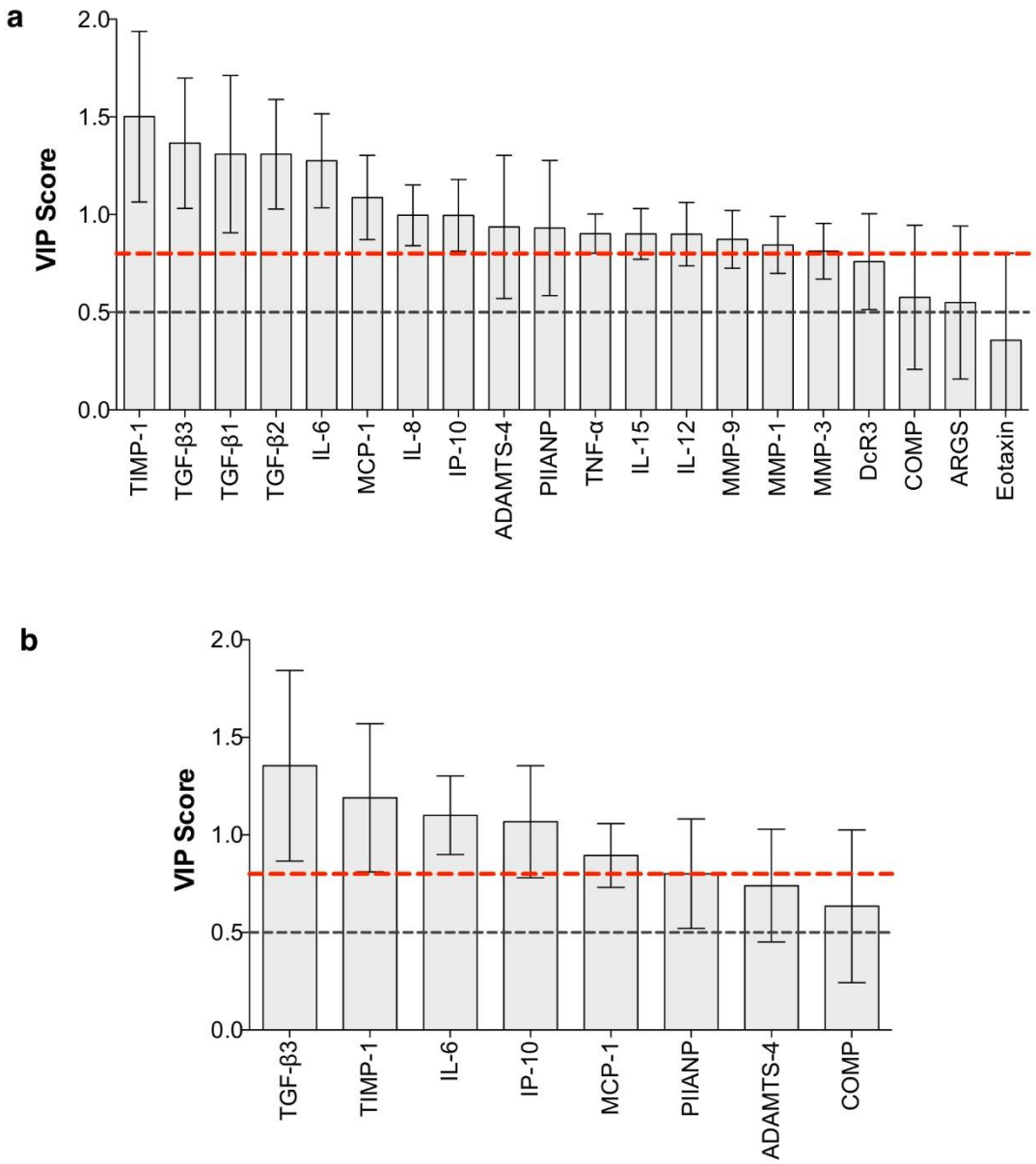

Fig. b. Variable Importance for Projection (VIP) Scores. a) Full model. b) Streamlined model. A measure of how much each X-variable (SF marker) contributes to the overall PLS-DA model. This includes both its importance to class separation (Y-variable) and its importance to modelling the latent structure of X-variables i.e. components. Markers with a VIP > 0.8 were considered important for the overall model; VIP between 0.8 and 0.5 considered potentially important, and VIP < 0.5 considered unimportant.

Model diagnostics

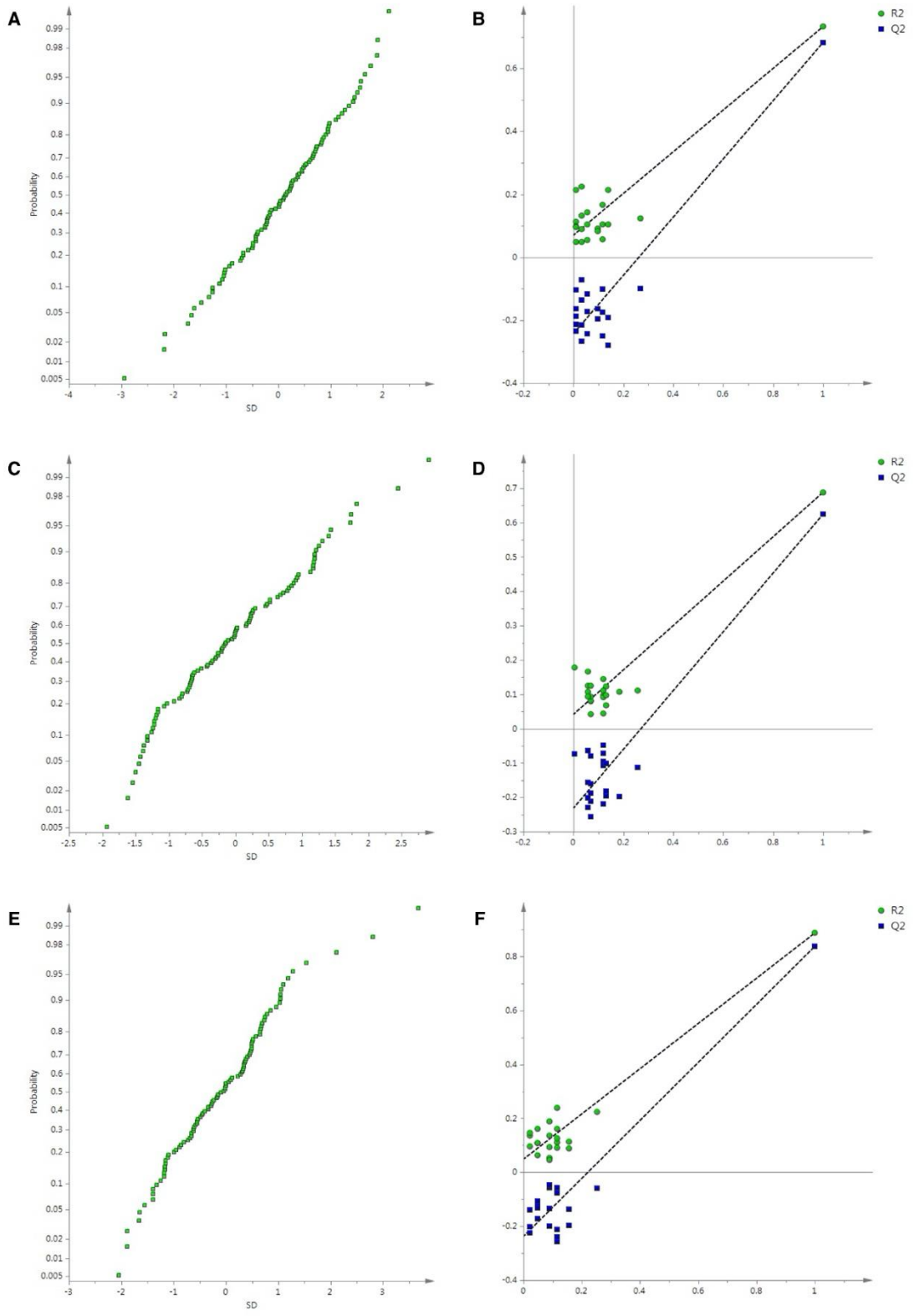

Fig. c. Model diagnostics for full PLS-DA model from 20 quantitative markers. Three-component model: R^2=0.770; Q^2=0.718. *Normal Probability Plot of Residuals* for a) end-stage knee OA, c) knee injury and e) inflammatory arthritis. *Permutations Plot* (20 permutations)

for b) end-stage knee OA, d) knee injury and f) inflammatory arthritis. See Supplementary Methods for further information on these model diagnostics.

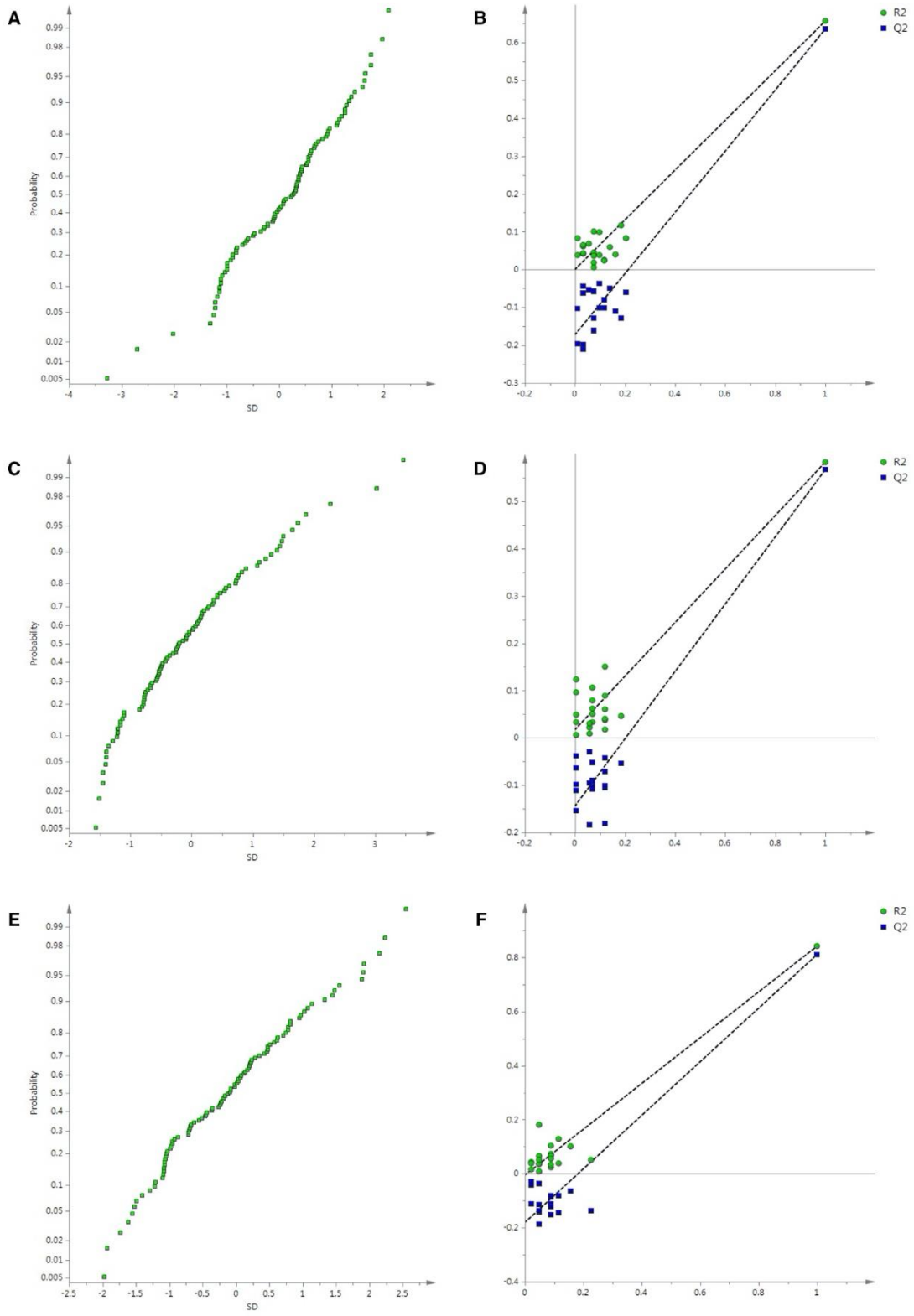

Fig. d. Model diagnostics for streamlined PLS-DA model from eight quantitative markers. Three-component model: R^2=0.770; Q^2=0.718. *Normal Probability Plot of Residuals* for a) end-stage knee OA, c) knee injury and e) inflammatory arthritis. *Permutations Plot* (20

permutations) for b) end-stage knee OA, d) knee injury and f) inflammatory arthritis. See Supplementary Methods for a further information on model diagnostics.

Model validation: clinical and radiological assessments

A clinical and radiological comparison of training-set and test patients with esOA is presented in **Supplementary Table v**.

Table v. Clinical and radiological assessments in patients with end-stage knee OA (esOA) - training versus test. Means (and 95% confidence intervals (CIs)) are given for age. Medians (and interquartile ranges (IQRs)) are given for Oxford Knee Score (OKS); OKS Pain and Function indices; and all radiological parameters. Radiological data shown are: Kellgren & Lawrence grades (KLG) in the worst compartment and total KLG score; total Osteoarthritis Research Society International (OARSI) score; OARSI joint space narrowing (JSN) subtotal; and OARSI osteophyte (OP) subtotal. One-way analysis of variance (ANOVA) with post-hoc Bonferroni multiple comparisons, or the Kruskall-Wallis test with post-hoc Dunn multiple comparisons were used as appropriate.

Variable	Training esOA	Test esOA
Patients, n	60	10
Sex, m:f	38:22	0:10*
Mean age, yrs (95% CI)	70.0 (67.7 to 72.3)	65.6 (58.1 to 73.2)
Median OKS (IQR)	19 (14.25 to 25.5)	18.5 (15.5 to 20.0)
Median OKS-Pain (IQR)	36 (21 to 46)	29 (17.75 to 44.25)
Median OKS-Function (IQR)	50 (35 to 64)	45 (35.0 to 55.0)
Median KLG Worst (IQR)	3 (3 to 4)	3 (2 to 3)
Median OARSI Total (IQR)	11 (7 to 14)	9 (5 to 11.5)
Median OARSI JSN (IQR)	3 (2 to 4)	3 (2 to 3)
Median OARSI OP (IQR)	7 (3.5 to 9)	5 (2 to 7.25)

*Statistically significant ($p < 0.05$).

Supplementary Discussion: Biological qualification

It must be emphasized that the multivariate models describe a pattern of biological features in combination with each other and must be taken as a whole. However, biological qualification of the findings requires examination of the individual markers.

Cytokines and inflammatory markers

As expected, pro-inflammatory and regulatory cytokines largely loaded towards inflammatory arthritis.[12,13] Similar findings have been reported previously.[14,15] Although there is substantial evidence for the role of inflammatory mediators and synovitis in the pathophysiology of OA, SF cytokines measurements in the multivariate models were more specific for inflammatory arthritis and important for discriminating against non-OA knee injury. Consequently, they may not be ideal OA biomarkers. Their individual utility would require reference ranges describing OA; below which OA is unlikely and above which inflammatory disease is more likely. The presentation of reference ranges has been intentionally avoided in this study because measured concentrations are at best quasi-quantitative being dependent on the precise assay used and numerous pre- and post-analytical factors. The diminished relative merit of cytokines as specific OA biomarkers is further suggested by the finding that only IL-6 was retained in the streamlined PLS-DA model with reduced levels characterizing knee injury. IL-6 may have a specific role in OA by inhibiting type II collagen gene expression (COL2A1) by chondrocytes.[16] However, the inflammatory cytokine IL-15 was a significant negative marker in the esOA fingerprint in the full PLS-DA model. These findings are consistent with a study by Scanzello et al[17] that demonstrated significantly lower SF IL-15 levels in esOA compared to early knee OA

(degenerate meniscal tears and medial KLG < 2). IL-15 is predominantly a membrane-bound mediator and increased intra-articular detection of the soluble form in early OA may represent reduced biological activity.

Eight of the 15 analytes that did not qualify for quantitative analysis were cytokines: four inflammatory cytokines (IL-1β, IL-2, GM-CSF, and LIGHT) and nearly all regulatory cytokines on the biological panel (IL-1RA, IL-2R, IL-4, and IL-10). One must be cautious in further interpretation of categorical data, and further sample analysis with more sensitive assays is warranted.

Chemokines

Chemokines play a crucial role in inflammation, but their actions are complex and not just restricted to recruitment of inflammatory cells. Accordingly, chemokines loaded towards inflammatory samples and quantification frequencies for non-quantitative chemokines were, on the whole, greatest in inflammatory samples. This pattern has been demonstrated previously.[18] MCP-1 and IP-10 were important markers in both the full and streamlined models being significant positive and negative elements, respectively, in the OA fingerprints. A recent comprehensive study by Harris et al[19] demonstrated that elevated SF MCP-1 levels in OA reduces the chondrogenic potential of synovial mesenchymal progenitor cells at a gene and protein level. A preliminary extensive multiplex analysis of SF by the authors showed significantly higher levels of MCP-1 in OA patients than cadaveric controls. The authors suggest that prolonged exposure to elevated MCP-1 "locks the joint in a vicious cycle of ineffective repair". In another wide-ranging multiplex analysis of SF from cadaveric normal controls and patients with mild/moderate OA or severe OA, Heard et al[20] found MCP-1 to be the most influential cytokine discriminating between normal and OA samples. Saetan et al[21] demonstrated that both SF and serum IP-10 were inversely correlated with increasing radiological severity in patients with knee OA, thereby offering an

explanation for this chemokine being an important negative feature in the esOA fingerprint. IP-10 is also a strong positive element in the parallel inflammatory fingerprint in both the full and streamlined multivariate models. Elevated IP-10 is known to be a feature of rheumatoid and psoriatic arthritis.[22-24] Eotaxin(-1) was not important in the multivariate models (VIP < 0.5) and did not feature in the biological fingerprints. Eotaxin has been induced in cytokine-activated chondrocytes and can stimulate chondrocyte MMP-3 expression and release.[25,26] However, there are conflicting reports in the literature with both elevated and unquantifiable SF eotaxin in OA patients compared to controls.[19,27] It is possible that increased clearance rates associated with greater synovitis from injury to OA to inflammatory knees masked any measured differences or the assay itself may have been unreliable. The quantification frequencies of RANTES were not significantly different between esOA and knee injury patients, which is consistent with a similar recent comparison.[28]

Growth factors

Osteophytosis is a cardinal feature of OA, and TGF-β has been regarded as central to osteophyte formation.[29] At first glance, it is surprising that all three TGF-β isoforms, and in particular TGF-β3, were negative elements of the esOA fingerprint. These results may be a consequence of loss of cartilage volume in esOA since a major source of TGF-β resides in the cartilage matrix.[30,31] Immunohistochemical analysis of OA cartilage in animal models have also demonstrated matrix TGF-β depletion.[32]

Recent work has illustrated the split personality of TGF-β that has complex pleiotropic actions in OA including both pro-inflammatory/catabolic and regulatory/anabolic capabilities depending on differential receptor pathway signalling.[33-36] TGF-β is required to maintain normal cartilage homeostasis and loss of signalling can lead to degeneration. In particular, TGF-β can regulate MMP and TIMP gene expression via Smad and activator

protein-1 (AP-1) signalling pathways and therefore reduced levels in OA may promote in MMP-mediated matrix degradation.[37] The results of this study favour the paradigm that OA is associated with reduced TGF-β anti-catabolic and reparative function. It is also possible that reduced matrix TGF-β in esOA is compounded by increased SF clearance via associated synovitis not found in knee injury. However, all three TGF-β isoforms strongly loaded towards inflammatory arthritis in the multivariate models. Synovitis, a cardinal feature of inflammatory joint disease, is likely to be a source of TGF-β, which has been shown to be elevated in the SF and synovium of inflammatory arthritis.[12,14,37]

BMPs have both anabolic and catabolic properties and play a role in osteophyte formation, cartilage repair, and remodelling.[38,39] Unfortunately, BMP- 2 and BMP-7 did not qualify for quantitative analysis, with BMP-7 virtually undetectable in SF samples from all study cohorts. However, other studies have successfully measured BMP-7 in OA and inflammatory SF using immunoassays and therefore the custom assay used in this study may have unsatisfactory analytical sensitivities.[15,40,41] This highlights the danger in directly comparing studies using different assays. Quantification frequency of BMP-2 was higher in inflammatory arthritis, followed by esOA and then knee injury samples, where no samples were > LOQ. Higher quantification frequency in inflammatory samples is most likely the result of BMP-2 expression by inflammatory synovium and stimulation by pro-inflammatory cytokines.[42-44] BMP-2 mRNA and protein expression has been shown to be reduced in healthy versus OA cartilage.[32,42,45]

Matrix enzymes

Matrix enzymes such as MMPs, aggrecanases, and TIMPs are key players on the frontline of the battle between matrix degradation and synthesis in OA where disequilibrium ultimately favours matrix destruction. Of the assayed MMPs, it was surprising that only MMP-9 (gelatinase-B) and MMP-13 (collagenase-3) featured in the multivariate models. The

importance of gelatinases in OA is unclear.[46] MMP-9 was an important variable in the full multivariate model, where it was a negative element in the OA fingerprint and a positive element in the parallel inflammatory fingerprint, but was not present in the streamlined model. The literature largely supports these findings with several studies demonstrating higher SF MMP-9, as well as MMP-1 (collagenase-1), MMP-3 (stromolysin-1), and TIMPs, in inflammatory arthritis than OA.[47-51] A recent study by Ryu et al[52] demonstrated significantly greater MMP-9 activity in SF from inflammatory knee conditions than OA using near infrared fluorescence probes and gelatin zymography. MMP-13 (collagenase 3) has a potent ability to degrade type II collagen and therefore its pivotal role in OA pathophysiology and potential as a therapeutic target have received considerable attention.[53] MMP-13 did not qualify for quantitative analysis, but was quantifiable in nearly 50% of esOA and inflammatory samples compared to only 5% of injury samples. Marini et al[54] also observed greater MMP-13 activity in patients with advanced cartilage degeneration compared to normal and early changes assessed by arthroscopy. In the absence of sufficient quantitative data, it is difficult to comment on differences in SF MMP-13 levels between OA and inflammatory arthritis and there are conflicting reports in the literature. Yoshihara et al[48] detected MMP-13 in significantly more knee SF samples from patients with rheumatoid arthritis than isolated OA using ELISA. However, Heard et al[51] demonstrated higher levels, with ELISA and Luminex, in knee SF of OA than rheumatoid arthritis patients on a variety of medications. One can speculate that MMP-13 would be an important positive element in the OA fingerprint of the quantitative multivariate models if a more sensitive assay was used.

Neither MMP-1 (collagenase-1) nor MMP-3 (stromolysin-1) was a significant component in any of the biological fingerprints. It must be stressed that immunoassays measure total MMP reflecting a combination of latent pro-MMP, active MMP, and both forms complexed to TIMPs and potentially other inhibitors. This study did not measure enzyme activity and, therefore, one cannot dismiss MMP-1 or MMP-3 in one of these specific forms as potential biomarkers.

ADAMTS-4 (aggrecanase-1) was a positive element in the esOA fingerprints of both the full and streamlined multivariate models. In the streamlined model it was also a negative element in the knee injury fingerprint. In addition to the finding that MMP-3 did not feature in the OA fingerprints, these results are consistent with the current consensus that the aggrecanases have a more specific and potent role in OA aggrecanase degradation than MMPs.[55-59] The expression of matrix enzyme inhibitors, such as TIMPs, is known to be elevated in OA as an attempt at maintaining matrix homeostasis. It was therefore not surprising that SF TIMP-1 concentrations mirrored the general pattern for MMPs, and TIMP-1 featured prominently in both the full and streamlined multivariate models being strongly associated with esOA and opposing knee injury. The TIMP- 1 immunoassay measures free and complexed TIMP-1 and SF TIMP-1 levels may represent a global approximation of matrix enzyme levels. Furthermore, there is increasing evidence that TIMPs, and in particular TIMP-1, have MMP-independent cytokine-like signalling activity involved in various processes including cell growth, apoptosis, and differentiation.[60]

Matrix metabolism

In both the full and streamlined multivariate models, PIIANP was a positive element in the esOA fingerprint and a negative element in the parallel knee injury and inflammatory fingerprints. PIIANP reflects synthesis of an embryonic form of type II collagen by chondrocytes that have undergone a phenotypic shift in OA cartilage.[61,62] At the time of writing, there were no previous studies reporting SF PIIANP levels in OA. Serum PIIANP concentrations have been reported to be significantly lower in patients with knee OA and rheumatoid arthritis than healthy controls, and there are conflicting reports regarding the association of serum levels with disease progression.[63-66] The findings in this study are more consistent with the expected paradigm and, furthermore, suggest SF PIIANP as a potential specific OA biomarker in its own right.

Both aggrecanase-generated aggrecan fragments and COMP release from degenerate cartilage matrix are considered potential biomarkers for OA. However, neither 374-ARGS aggrecan neo-epitope or COMP was a significant element in the detailed OA fingerprint. ARGS was intriguingly a positive element in the parallel knee injury fingerprint and this was echoed in the finding that SF ARGS concentrations were significantly greater in knee injury than esOA samples. SF COMP levels were lowest in inflammatory samples and there was a trend to be greatest in knee injury samples. Although COMP qualified for the streamlined multivariate model, it was only significant as a negative element for the inflammatory fingerprint. These findings are unexpected since SF MMP and ADAMTS-4 concentrations were lowest in knee injury samples. Furthermore, the results question the true biological nature of macroscopically normal cartilage in knee injury patients. The mechanical and biological implications of knee injury for the risk and progression to OA are well documented in the literature. Swärd et al[67] demonstrated elevated SF 393-ARGS aggrecan neo-epitope levels in acutely injured knees up to 23 days after injury in comparison to healthy reference knees. Larsson et al[68] reported SF 393-ARGS aggrecan neo-epitope levels in injured knees (ACL rupture +/- meniscal tear) with low OA scores were higher than in patients with knee OA within 12 weeks from injury and were equivalent after 12 weeks from injury. However, in the present study SF 374-ARGS levels remain higher in the knee injury than esOA patients at a median 6.5 months (IQR 4 to 9.75) post index injury. The authors also suggest that elevated SF ARGS is due to aggrecanase activity against existing matrix aggrecan. In severe and esOA, there is reduced existing cartilage matrix volume and therefore relatively lower ARGS neo-epitope release may be expected despite higher aggrecanase levels. This is supported by evidence that SF proteoglycan fragment concentration decreases with increasing radiological OA severity which is associated with lower cartilage and proteoglycan mass.[69]

The findings for COMP are more difficult to reconcile. Kokebie et al[15] were unable to demonstrate any differences in SF COMP concentration between patients with knee OA,

rheumatoid arthritis, or asymptomatic donors. El-Arman et al[70] reported higher SF COMP concentrations in patients with knee OA than traumatic effusion; furthermore there was a positive correlation with both radiological and MRI severity scores. The traumatic effusion group may represent a different population to the present knee injury cohort since they had no evidence of meniscal injury and the authors make no comment on possible ACL injury. Consistent with the results of the present study, Lohmander et al[71] demonstrated SF COMP to be higher in knees with meniscal injury or ACL rupture +/- meniscal injury than primary OA. The authors also report a trend for SF COMP levels to decrease with increasing knee OA severity. As with ARGS aggrecan neo-epitope, loss of cartilage matrix volume in severe and esOA means there is less COMP for release. COMP that has already been released may have been degraded by MMPs and aggrecanase and therefore not detected by the immunoassay.[72-76] The negative association of SF COMP with the inflammatory cohort may be also be a consequence of group heterogeneity and disease-modifying treatments.[77,78] Furthermore, increased molecular clearance associated with greater synovitis in esOA and inflammatory arthritis may contribute to lower measured levels of both ARGS aggrecan neo-epitope and COMP compared to knee injury.

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