The absence of detectable ADAMTS-4 (aggrecanase-1) activity in synovial fluid is a predictive indicator of autologous chondrocyte implantation success.

Abstract

Background: Autologous chondrocyte implantation (ACI) is used worldwide in the treatment of cartilage defects in the knee. Several demographic and injury specific risk factors have been identified that can affect the success of ACI treatment. However, the discovery of predictive biomarkers in this field has thus far been overlooked.

Hypothesis/Purpose: The purpose of this study was to identify potential biomarkers in synovial fluid (SF) and plasma that can be used in the pre-operative setting to help optimise patient selection for cell-based cartilage repair strategies.

Study design: Case series; Level of evidence, 4.

Methods: Fifty four ACI-treated patients were included. Cartilage oligomeric matrix protein (COMP), hyaluronan, soluble CD14 levels and ADAMTS-4 activity in synovial fluid and COMP and hyaluronan in plasma were measured. Baseline and post-operative functional outcome was determined using the patient-reported Lysholm score. To find predictors of post-operative function, linear and logistic regression analyses were performed. The dependent variables were baseline and post-operative Lysholm score, the independent variables were patient age and body mass index, defect location, defect area, having a bone-on-bone defect, type of defect patch type (periosteum or collagen), requirement of an extra procedure and baseline biomarker levels.

Results Mean baseline Lysholm score was 47.4(+/-17.0), which improved to 64.6(+/-21.7) post-operatively. The activity of ADAMTS-4 in synovial fluid was identified as an independent predictor of post-operative Lysholm score. Indeed, simply the presence or absence of ADAMTS-4 activity in synovial fluid appeared to be the important predictive factor (determined by contingency analysis). Other predictive factors were baseline Lysholm score, age at ACI and the defect patch type used.

Conclusions The absence of ADAMTS-4 activity in the synovial fluid of joints with cartilage defects may be used in conjunction with known demographic risk factors in the development of an ACI treatment algorithm to help inform the preclinical decision.

What is known about the subject: There are no known predictive biomarkers for ACI.

What this study adds to existing knowledge: This study has identified the first biological predictor for ACI, which could be used in deciding the best treatment.

Introduction

A recent white paper on how to move forward with cell-based advanced therapies has highlighted the need for improved predictive preclinical efficacy testing within Europe[15]. Hence, the refinement and optimisation of cell therapy protocols for increased efficacy in the treatment of early osteoarthritis (OA) is more relevant than ever before. Some patient demographics have been linked to failure, including age, gender, body mass index (BMI) and the size of cartilage lesion[19,20,41] and are used when deciding on ACI treatment. Currently there are no wet biomarker tests available to clinicians that can accurately and reproducibly predict the outcome of cell therapies for cartilage repair. There is, therefore, a clinical and financial need for reproducible pre-operative biomarkers assays, to be used in conjunction with known demographic risk factors, to help the clinician decide if a patient should be considered suitable for treatment using these interventions.

Wet biomarkers derived from body fluids (e.g. synovial fluid and blood plasma) represent an attractive option for the pre-operative screening of patients. In addition, such markers could also enable longitudinal analyses to monitor treatment outcome or act as surrogate outcomes, or help to elucidate the reasons why some patients benefit from treatments when others fail to do so. Finally, the characterisation of specific biochemical and or cellular changes in these fluids, associated with treatment failure or success, may help to identify markers to target and improve the therapeutic effect[16].

Several compounds found in the blood and synovial fluid have been associated with cartilage injury and OA progression, including matrix molecules and enzymes associated with cartilage degradation such as hyaluronan (HA)[36], cartilage oligomeric matrix protein (COMP)[7], chondroitin sulphate (CS)[3], aggrecanase-1/aggrecanase-2 (ADAMTS-4/ADAMTS-5)[18,23,46] and matrix metalloproteinases (MMPs)[1]. In addition, other molecules may be suitable as biomarkers such as those associated with inflammation, including soluble CD14 (sCD14)[11,26]. The main objective of this study was to begin the process of establishing a reliable panel of biochemical markers that

could be used in the pre-operative setting, to optimise treatment selection for cartilage defects.

Materials and Methods

Patients

Following approval from [redacted] and with informed consent, synovial fluid and plasma was obtained pre-cell implantation from all consented patients treated with autologous chondrocyte implantation (ACI) at our centre from 2007 to 2012 inclusive. ACI is a two-stage procedure, with stage I an arthroscopic cartilage harvest and cells being implanted during an arthrotomy at stage II. The procedure was performed using culture-expanded chondrocytes, as described previously[4,6,32]. The indication for ACI in all of the patients included in this study was the presence of a focal cartilage defect in the knee. All patients included in the study had received either debridement or microfracture as a previous surgical treatment for these defects and as such none of the patients could be described as acutely injured. At baseline (pre-ACI), we recorded age, gender, BMI and the functional status as characterised by the modified Lysholm scale (score range is 0-100, where the maximum score is 100, which denotes an 'excellent' functioning knee joint)[38]. We further recorded the defect location and size, whether the defect was 'bone on bone' and whether the patient underwent additional procedures including ligament reconstruction, meniscal replacement surgery or osteotomy. We also recorded the type of material used to cover the defect before cell implantation i.e. autologous periosteum or porcine collagen membrane (Chondro-Gide®; Geistlich Ltd, Manchester, UK). A patient was described as a 'responder' or a 'non-responder' based on the change in 1-year post-operative Lysholm score. Responders were patients who had improved by at least 10 points, which is comparable to the published minimum clinically important difference for 100 point functional knee scores reported in other studies[14,34,37].

Synovial fluid and plasma collection and storage

To collect synovial fluid, patients' knee joints were injected with 20mls of saline followed by 20 cycles of extension and flexion prior to intra-articular aspiration. We

have been collecting synovial fluid from knee joints for biomarker analyses for the last 15 years. This process of collection has been optimised for ease of synovial fluid volume retrieval by the clinician and to ensure, as much as possible, consistency between samples. Blood plasma was obtained by venepuncture at the time of ACI. Synovial fluid and blood were centrifuged at 600g for 15 minutes at 4^0C. The synovial fluid and blood plasma were then divided into aliquots and stored in liquid nitrogen prior to biomarker analyses. The dilution of synovial fluids was accounted for by normalising synovial fluid biomarker values to the urea concentration in blood plasma. In brief, urea concentrations were measured in the synovial fluid and in the blood plasma (which were harvested at the same time) and a dilution factor was calculated for the synovial fluid based on the assumption that the urea concentration is equivalent in plasma and synovial fluid, a previously reported methodology[21]. COMP, HA, sCD14 levels and ADAMTS-4 activity in synovial fluids were calculated by multiplying assay derived concentrations by the dilution factors obtained from the urea analyses.

COMP and HA quantification in synovial fluid and matched blood plasma

COMP levels in synovial fluid and plasma were determined using an enzyme-linked immunosorbant assay (ELISA) (BioVendor Laboratory Medicine, Modrice, Czech Republic). For calculations of COMP concentration, the logit log function was used to linearise standard curves. HA levels in synovial fluid and plasma were measured using an enzyme-linked protein binding assay (Corgenix, Broomfield, CO). Third-order polynomial regression was used to generate the best-fit curve to calculate the concentration of HA in patient samples. For COMP and HA assays all commercially provided quality controls were within accepted limits. The limit of detection (LoD) for COMP and HA were calculated to be 0.1ng/ml and 17.8ng/ml respectively. The inter- and intra-assay co-efficient of variance was 7.1% and 1.6% for COMP assays and 19.1% and 4.5% for HA assays, respectively.

sCD14 quantification in synovial fluid

sCD14 was measured in synovial fluid samples using the Human sCD14 Quantikine® ELISA (R&D Systems, Minneapolis, MN). For calculations of sCD14 concentration the logit log function was used to linearise standard curves. The LoD was calculated to be 141pg/ml. The inter- and intra-assay co-efficient of variance was 9.0% and 4.3%, respectively.

ADAMTS-4 activity quantification in synovial fluid

An end-point fluorometric substrate assay (SensoLyte®520 Aggrecanase-1 Assay Kit, AnaSpec, Fremont, CA) was used to measure ADAMTS-4 activity in synovial fluid samples. The kit contains an internally quenched 5-carboxyl fluorescein (FAM)/ tetramethylrhodamine (TAMRA) fluorescence resonance energy transfer (FRET) substrate which is optimised to specifically detect ADAMTS-4 activity (down to 4ng/ml). For calculations of ADAMTS-4 activity linear standard curves were constructed by plotting relative fluorescent units (RFU) versus the concentration of ADAMTS-4 standards (AnaSpec). The LoD was calculated to be 1.4ng/ml, with inter- and intra-assay co-efficient of variances being 12.3% and 1.7%, respectively.

Statistical analysis

The distributions of all continuous variables were investigated using quantile-quantile (QQ) plots. These showed that age, BMI and the Lysholm scores followed a normal distribution, whereas defect area and the levels of all biomarkers followed a log-normal distribution.

Some biomarker levels were below the assay detection limit ('non-detects' or 'censoring'), in those cases, imputed biomarker levels were used that minimised bias[29]. The imputation was based on a larger set of biomarker samples, comprising all samples from the current study augmented with 66 samples from 53 patients collected at least 1 year post-operatively. When fewer than 10% of the specimens were a non-detect for a specific biomarker, an imputation value of $(1/\sqrt{2})$ times the LoD was used. At non-detect levels above 10%, we imputed using the expected value of the biomarker level. These values were calculated using a censored log-normal distribution through

all biomarker levels above the detection limit[28]. However, no imputation was needed for such a biomarker when it was the dependent variable in the analysis; instead we used a censored regression analysis[12]. For patients with two baseline specimens i.e. a Stage I and a matched Stage II sample, the mean biomarker level both was used.

To find predictors of post-operative functional status, a univariable linear regression analysis was performed using the post-operative Lysholm score as the dependent variable and including the baseline Lysholm score as a covariate. Independent variables were patient age at the time of ACI, BMI, defect location, defect area, having a bone-on-bone defect, type of defect patch type (periosteum or collagen), requirement of an extra procedure and the baseline level of the five synovial fluid biomarkers (HA, ADAMTS-4, COMP and sCD14) and the two plasma biomarkers (HA and COMP). A multivariable linear regression analysis was then performed based on all predictors with a univariable *p*-value below 0.15. Further univariable and multivariable linear regression analyses were performed to identify predictors of ACI outcome based on the responder definition described above, namely assuming that responders were patients with an improvement of at least 10 points[14,34,37]. These analyses were performed as logistic regression analyses, following the same methodology as outlined above for the linear regression analyses. The resulting model was then internally validated (see Appendix).

For those biomarkers that were identified as having a predictive value we also plotted their baseline levels versus the difference from baseline Lysholm score in order to determine whether merely the presence of the biomarker or the quantity measured is important for predicting response. This was examined further in a 2-way contingency table analysis (Fisher's exact test). Finally, for all biomarkers with a *p*-value in the multivariable linear or logistic regression analysis below 0.15, further linear regression analyses were performed to determine predictors of the biomarker levels amongst the other variables examined, i.e. all demographic and defect characteristics as well as the levels of the other biomarkers in the same fluid compartment (synovial fluid or plasma).

All regression analyses were performed using R vs 3.0.3 (R Foundation for Statistical Computing, Vienna, Austria), using the packages ‘envStats’, ‘distrEx’, ‘rms’ and ‘lme4’. All analyses used the appropriate transformation identified from the QQ plots, and a *p*-value below 0.05 was assumed to denote statistical significance.

Results

Patients

Fifty four patients undergoing ACI were included in this study with a mean baseline clinical Lysholm score of 47.4(+/-17.0) which improved to 64.6(+/-21.7) at post-operative assessment (2.2+/-3.0 years post-treatment). Seventy seven percent of patients showed an increase in Lysholm score at this time point, with 59% improving at least 10 points, these were classed as responders in subsequent analyses. The study comprised samples from 38 male and 16 female patients, aged between 17 and 61 years at the time of ACI. An overview of the demographic variables and defect characteristics analysed are detailed in Table 1.

Table 1: Overview of demographic and defect variables.

Variable	Mean (SD), Median (IQR) or n (%)	Range	Number of non-detects (%)
Age (years)	35.0 (10.2)	17-61	
Gender			
Female	16 (30%)		
Male	38 (70%)		
BMI	27.4 (4.1)	20-37	
Defect location			
Medial femoral condyle	35 (65%)		
Lateral femoral condyle	5 (9%)		
Patella	5 (9%)		
Trochlea	5 (9%)		
Trochlea & patella	1 (2%)		
Tibial plateau	3 (6%)		
Defect area (cm^2)	3.6 (1.5-6.0)	0.02-43.2	
Bone-on-bone			
No	41 (95%)		
Yes	2 (5%)		
Patch type			
Collagen	47 (87%)		
Periosteum	7 (13%)		
Additional procedure			
No	32 (59%)		
Yes	22 (41%)		
Pre-op Lysholm	47.4 (17.0)	13-92	
Post-op Lysholm	64.6 (21.7)	29-100	
Synovial fluid markers			
HA (mg/ml; $\times 10^6$)	1.7 (1.1-3.1)	0.4-9.1	0 (0%)
ADAMTS-4 (ng/ml)	0.40 (0.40-41.5)	0.40-416	54 (71%)
COMP (µg/ml; $\times 10^4$)	9.3 (4.0-16.1)	0.03-42.6	0 (0%)
sCD14 (ng/ml; $\times 10^2$)	8.8 (6.5-12.9)	0.31-29.5	1 (1%)
Plasma markers			
HA (ng/ml)	17.4 (7.3-28.0)	0.0-79.3	0 (0%)
COMP (ng/ml; $\times 10^2$)	7.0 (4.8-13.3)	1.0-27.5	0 (0%)

SD is Standard Deviation; IQR is Interquartile Range (lower to upper quartile). The values for non-detects were imputed when calculating median biomarker levels.

Biomarker levels

From the 54 patients included, 76 fluid samples were collected: 47 at stage I (pre-cartilage harvest) and 29 at stage II (pre-cell implantation, 3-4 weeks later). In 11 patients a sample was collected at both stages. The mean synovial fluid dilution factor (calculated using blood plasma urea content)[21] was 4.7+/-3.6 and in the responder and non-responder groups the dilution factor was 4.4+/-3.1 and 4.9+/-3.7, respectively. For

two biomarkers (sCD14 and ADAMTS-4), we found samples with undetectable levels. In order to use the biomarker variables with these 'non-detects' as predictors in a regression analysis, imputed values were used for all non-detect cases. For sCD14 levels, where sCD14 was below the detection limit in only one sample, the imputed value was taken equal to $(1/\sqrt{2})$ times the LoD of 0.14ng/ml (0.10ng/ml). The ADAMTS-4 determinations had 71% of non-detects and its lower detection limit (LDL) was 3.7ng/ml. A censored log-normal distribution was fitted to the ADAMTS-4 data, giving a mean of -1.6 and an SD of 4.4. The expected value of ADAMTS-4 under the above log-normal distribution and the condition that the value was below 3.7ng/ml was 0.40ng/ml. This value of 0.40 was used to impute the non-detect cases. Mean baseline biomarker levels after imputation are summarised in Table 1.

Regression analyses of predictors of post-operative Lysholm score following ACI treatment.

When using post-operative Lysholm as the main outcome, and including baseline Lysholm as a covariate, univariable linear regression models found two variables potentially associated with better outcome, namely lower age and lower ADAMTS-4 activity (Table 2A). A higher baseline Lysholm, lower age and lower ADAMTS-4 activity all proved to be significantly associated with a higher 1-year Lysholm score in a multivariable regression analysis (Table 2B).

Table 2A: Univariable linear regression analysis of predictors of post-op Lysholm score.

Variable	Regression coefficient (SEM)	Partial R^2	*p*-value
Age	-0.50 (0.24)	0.08	0.04
Gender (Male)	8.1 (5.8)	0.04	0.17
BMI	-0.36 (0.78)	0.00	0.64
Defect location	-	0.09	0.49
Defect area (log)	0.07 (2.10)	0.00	0.97
Bone-on-bone (Yes)	-10.8 (14.1)	0.00	0.45
Additional procedure (Yes)	0.79 (5.2)	0.00	0.88
Patch type (Periosteum)	-7.4 (7.5)	0.02	0.33
Synovial fluid markers (baseline levels)			
HA (log)	2.1 (3.4)	0.00	0.55
ADAMTS-4 (log)	-2.0 (1.0)	0.08	0.045
COMP (log)	1.3 (2.1)	0.01	0.53
sCD14 (log)	-1.7 (3.3)	0.01	0.59
Plasma markers (baseline levels)			
HA (log)	-0.54 (0.58)	0.02	0.36
COMP (log)	1.3 (3.5)	0.00	0.71

All analyses included the baseline Lysholm score as an independent variable. SEM is Standard Error of the Mean. The partial R^2 is a measure of the correlation of each variable with the post-op Lysholm score while eliminating the influence of baseline Lysholm score.

Table 2B: Multivariable regression analysis of predictors of post-op Lysholm score.

Component	Regression coefficient (SEM)	R^2	*p*-value
Total model	-	0.40	<0.001
Baseline Lysholm score	0.74 (0.14)	0.35	<0.001
Age	-0.49 (0.23)	0.08	0.04
ADAMTS-4 level (log)	-1.95 (0.94)	0.08	0.04

SEM is Standard Error of the Mean. The R^2 for each separate model component is its partial R^2, the R^2 when eliminating the influence of the other components.

Regression analyses of predictors of a 10 point improvement in Lysholm score following ACI treatment (responder analysis).

Amongst our 54 patients, 32 had an improved Lysholm (at least 10 points) after 1 year and were classed as responders, whereas 22 did not show such an improvement and were classed as non-responders. Univariable logistic regression analyses found that a lower age, collagen patch type and lower ADAMTS-4 activity in synovial fluid predicted a positive response (Table 3A). A multivariable logistic regression model of potential

predictors demonstrated that age, patch type and ADAMTS-4 levels were again predictive (Table 3B and Figures 1A-C).

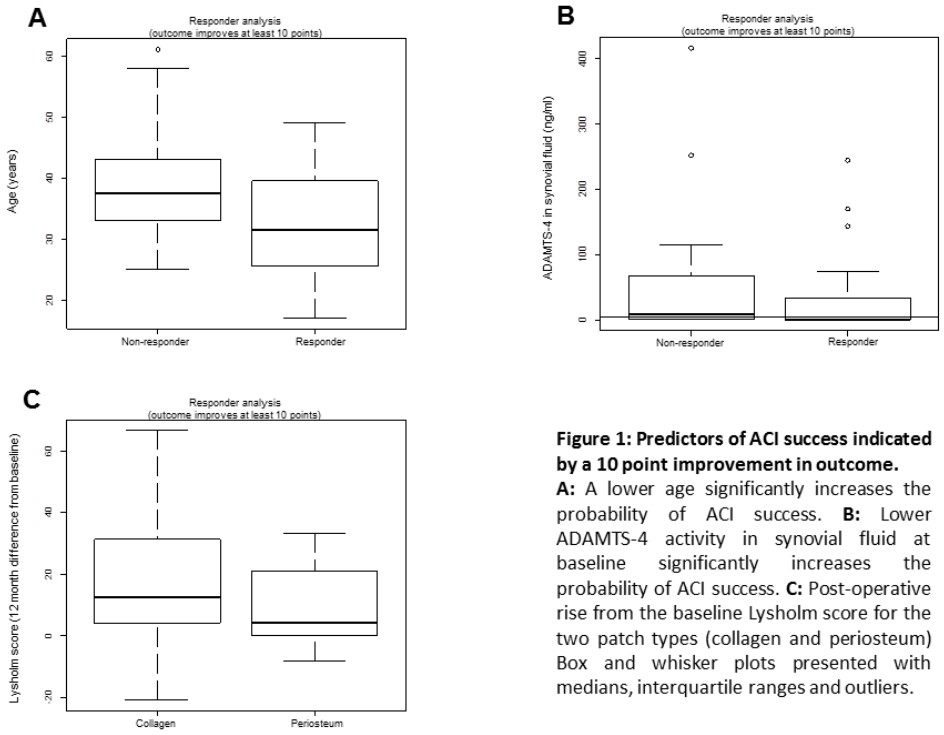

Figure 1: Predictors of ACI success indicated by a 10 point improvement in outcome.
A: A lower age significantly increases the probability of ACI success. **B:** Lower ADAMTS-4 activity in synovial fluid at baseline significantly increases the probability of ACI success. **C:** Post-operative rise from the baseline Lysholm score for the two patch types (collagen and periosteum) Box and whisker plots presented with medians, interquartile ranges and outliers.

Plotting baseline ADAMTS-4 levels versus the change in Lysholm score from the baseline score suggested that the main predictive aspect of ADAMTS-4 was its presence rather than its quantity (data not shown). A simple 2-way contingency table analysis confirmed that this was indeed the case (Table 3C; p=0.05, Fisher's exact test). The odds ratio (OR) of 0.33 indicates that when ADAMTS-4 activity was detectable the odds of being a responder were 3 times smaller than when ADAMTS-4 activity was not detectable.

Table 3A: Univariable logistic regression analysis of predictors of an increase in Lysholm score of at least 10 points.

Variable	Odds Ratio (SEM)	C-index (concordance)	*p*-value
Baseline Lysholm score	0.98 (0.02)	0.57	0.31
Age	0.92 (0.03)	0.69	0.01
Gender (Male)	1.35 (0.82)	0.52	0.75
BMI	0.94 (0.08)	0.51	0.59
Defect location	-	0.62	1.00
Defect area (log)	0.90 (0.21)	0.58	0.65
Bone-on-bone (Yes)	0.00 (0.01)	0.56	0.77
Additional procedure (Yes)	0.99 (0.56)	0.50	0.98
Patch type (Periosteum)	0.23 (0.20)	0.58	0.10
Synovial fluid markers (baseline)			
HA (log)	1.43 (0.54)	0.57	0.34
ADAMTS-4 (log)	0.82 (0.09)	0.63	0.08
COMP (log)	0.75 (0.21)	0.61	0.30
sCD14 (log)	0.72 (0.29)	0.67	0.42
Plasma markers (baseline)			
HA (log)	0.80 (0.27)	0.56	0.51
COMP (log)	0.92 (0.35)	0.52	0.83

SEM is Standard Error of the Mean.

Table 3B: Multivariable regression analysis of predictors of an increase in Lysholm score of at least 10 points.

Component	Odds Ratio (SEM)	C-index (concordance)	*p*-value
Total model	-	0.78	0.002
Age	0.91 (0.04)		0.01
Patch type (Periosteum)	0.15 (0.15)		0.06
ADAMTS-4 level (log)	0.77 (0.10)		0.05

SEM is Standard Error of the Mean.

Table 3C: Two-way contingency table of the relationship between detectable levels of ADAMTS-4 and response to ACI treatment.

Responder	Non-detectable ADAMTS-4	Detectable ADAMTS-4	OR	*p*-value
Yes	23	9	0.33	0.05
No	10	12		

OR is Odds Ratio. *p*-value based on Fisher's exact test.

Predictors of ADAMTS-4 activity

Finally, using a censored regression model (Table 4), we have found that none of the demographic or clinical baseline parameters were predictive of ADAMTS-4 activity; only higher sCD14 levels in synovial fluid were significantly associated with ADAMTS-4 activity level (p=0.003).

Table 4: Univariable censored regression analysis of predictors of log-transformed ADAMTS-4 levels.

Variable	Regression coefficient (SEM)	*p*-value
Concurrent Lysholm	0.01 (0.03)	0.65
Age	0.01 (0.05)	0.88
Gender (Male)	-0.76 (1.10)	0.49
BMI	-0.04 (0.17)	0.82
Defect location	-	0.90
Defect area (log)	-0.25 (0.39)	0.53
Bone-on-bone (Yes)	1.03 (2.68)	0.70
Additional procedure (Yes)	0.48 (1.05)	0.65
Synovial fluid markers		
HA (log)	0.58 (0.70)	0.40
COMP (log)	0.68 (0.55)	0.21
sCD14 (log)	2.73 (1.09)	0.01

SEM is Standard Error of the Mean.

Discussion

The over-arching aim of this study was to begin the process of developing a panel of biomarkers that could help clinicians to successfully treat more patients at an early stage of joint damage with less invasive treatments and thereby reducing the number of patients who might otherwise progress to end-stage OA and require knee replacement[10,12]. To this end, we have attempted to derive a clinical prediction model by, performing a series of univariable and multivariable linear and logistic regression analyses to predict clinical outcome of ACI from biomarker levels and demographic variables. Important criteria in such models are the precision of regression coefficients and the ability to perform well over a broad range of samples. The emphasis on precision implies that hypothesis testing is less relevant, and, as a consequence, no type I error adjustment was needed despite considering multiple independent variables[40]. Ultimately, our model requires validating with a further sample of patients. The internal validation we performed (see Appendix) is a second-best option, but its results do suggest that our prediction model will indeed perform well with future samples.

When using post-operative Lysholm as the outcome, and including baseline Lysholm as a covariate, lower age at ACI and lower ADAMTS-4 activity level were identified as promising predictors of better outcome, and both proved significant in a multivariable analysis. When an improvement in Lysholm score of at least 10 points was the definition of an ACI responder, which is an accepted clinical threshold in other published studies[14,34,37], three potential predictors were identified, namely a lower age, the patch type used in the second stage of the procedure (collagen) and the absence of ADAMTS-4 activity in synovial fluid. It is not too surprising that age at ACI and patch type have been identified in these analyses as potential predictors of ACI success in the clinic. Our group and others have already indicated that age and patch type have predictive value for ACI in terms of outcome and quality of repair cartilage

307 respectively[13,17,20,25,31]. However, the fact that we have identified ADAMTS-4 activity in
308 synovial fluid as a potential predictive indicator for ACI is completely novel. It is known
309 that ADAMTS-4 activity is elevated in the synovial fluid of arthritic joints[23,46]. Together
310 with ADAMTS-5 (another member of the ADAMTS gene family), ADAMTS-4 has been
311 shown to accelerate the loss of aggrecan from cartilage, which is a major contributing
312 factor in the progression of OA[2,35,39]. An alternatively spliced variant of the enzyme
313 (ADAMTS-4_v1), which lacks the spacer domain and to date has only been found to be
314 produced by osteoarthritic synovium cells, is suggested to contribute similarly to the
315 loss of aggrecan from the superficial zone of OA cartilage[43,44]. Elevated ADAMTS-4
316 activity in the synovial fluid of patients that have a poorer outcome following ACI might
317 therefore suggest that OA has already progressed too far, meaning that their joints are
318 less likely to benefit from standard ACI treatment. Interestingly, we have also shown
319 that none of the patient demographic or injury-associated variables examined in this
320 study could help to predict ADAMTS-4 activity. Hence, ADAMTS-4 activity is an
321 independent predictor of post-operative Lysholm score. Previous work that was
322 conducted on a larger cohort of patients which included a proportion with end-stage
323 OA[33], also investigated the relationship between demographic variables and ADAMTS-
324 4. That study found significantly higher levels of ADAMTS-4 activity in the synovial fluid
325 of older patients and that ADAMTS-4 activity in synovial fluid correlates with the level of
326 effusion noted in the knee. The finding of no association with age in the present study
327 is probably most readily explained by the smaller sample size. When exploring
328 biomarker associations we have shown that sCD14 levels are directly related to
329 ADAMTS-4 activity in synovial fluid, this finding is not too surprising as CD14 positive
330 macrophages are known to drive synovial ADAMTS-4 synthesis as part of the
331 destructive and inflammatory responses characterised in OA[5].

332 We have endeavoured to include as much patient demographic and injury
333 information as available in our regression analyses, because it is known that patient
334 age, sex, BMI and size and location of the defect can impact outcome following ACI[4,13]

and may also affect biomarker levels in synovial fluid. However, it was not always possible to obtain all information for all patients, especially in a retrospective study such as this. For example, the presence of additional pathologies which are likely to predispose to ACI failure were not always described at arthroscopy; these might have included the presence of synovitis, meniscal tears or the state of the subchondral bone. In addition, since our retrospective sample cohort only had matched data at both stages from 11 patients, it was not possible to accurately determine the impact of the Stage I procedure on biomarker levels at Stage II. We acknowledge that this missing information is a limitation of the study. Whilst studying biomarkers in synovial fluid can provide more information on the biology of degenerative joint disease than measuring their levels systemically, the ability to measure levels in blood plasma or serum is much more practical for widespread use in the clinic. Alternative methods for detection of aggrecanase activity have shown promise for correlating levels in synovial fluid and blood where aggrecan breakdown products are measured as opposed to ADAMTS-4 activity[22,23]. We aim to evaluate these methodologies for our ACI patient cohort in future studies using preparations of blood serum and plasma.

To our knowledge, the study of biomarkers in synovial fluids and bloods from patients treated for cartilage injuries is limited to three published studies[27,42,45], only two of which have analysed biomarkers in conjunction with outcome following ACI treatment. Nganvongpanit *et al* monitored serum levels of CS and HA in ten dogs following ACI and drilling, with levels of CS in serum correlating negatively with quality of repair[27]. Vasara *et al.* followed ten patients pre-ACI to 1 year post-ACI, monitoring MMP3 and IGF-1, and saw higher levels of these at both time points compared to non-treated controls[42]. The sample size in our study was larger in comparison, but was still relatively small (54 patients), which limited the power of our analyses. The coefficient of determination (R^2) between predictor and outcome would need to be at least 0.13 to be significant at the p=0.05 level, assuming 80% power. This threshold could explain why our analysis failed to identify demographic variables, such as gender or BMI found to

be predictive in a previous study which has assessed aspects of cell quality in predicting the clinical outcome of ACI using slightly more patients (n=80)[30].

The high detection limit of two assays (ADAMTS-4 and sCD14) prevented determining biomarker levels in all patients in our study. When the biomarker is the independent variable in a regression analysis this poses no problem because a censored regression analysis can be used[33]. However, when the biomarker is a dependent variable another approach is needed. Because sCD14 measurements were below the detection limit in only one sample, a simple and widely-used imputation method (LDL/$\sqrt{2}$) could be used[28]. However, ADAMTS-4 activity was below the detection limit in 54/76 samples (71%). With such a large proportion of left-censored data the simple imputation method would give biased results[24,28]. Simply omitting all cases with missing data would be highly inefficient because it discards all information contained in the left-censored observations[24,28]. The method we chose (fitting a censored log-normal distribution to all biomarker values above the detection limit) has been found to provide unbiased estimates of the regression coefficients in comparative tests[24,28]. This method performs best when the ADAMTS-4 data follows a censored log-normal distribution and data from an earlier study in our centre with three times as many samples above the detection limit found no evidence against the assumption of a log-normal distribution[33]. We therefore believe that the statistical methodology we used to handle the left-censored data below the detection limit conforms to best current practice.

In summary, through this report, we have identified that ADAMTS-4 activity in synovial fluid shows promise as a predictive biomarker to improve ACI patient selection for cell therapy. We suggest that ADAMTS-4 activity levels in the synovial fluid of joints with cartilage defects may be used to help identify patients who will have a poorer outcome following conventional ACI treatment. Specifically, our results and analyses demonstrate that it is the absence of ADAMTS-4 activity preoperatively in synovial fluid that has the greatest value in predicting a positive response to ACI. In combination with

other known predictors of ACI response such as age, gender, BMI and defect characteristics, the absence of detectable ADAMTS-4 in synovial fluid could be used when deciding the best treatment for cartilage defect patients. With that information, a treatment algorithm could be developed containing demographic risk factors and a panel of biomarker characteristics which will help to inform the preclinical decision. In addition, ADAMTS-4 activity is in itself a likely therapeutic target for combined biological treatments. Concurrent administration of molecules that specifically inhibit aggrecanases[8,9] with biological treatments may improve outcomes in patients with high levels of ADAMTS-4 activity in their joints, who would otherwise be less likely to benefit from standard ACI treatment.

References

1. Bay-Jensen AC, Liu Q, Byrjalsen I, Li Y, Wang J, Pedersen C, Leeming DJ, Dam EB, Zheng Q, Qvist P, Karsdal MA. Enzyme-linked immunosorbent assay (ELISAs) for metalloproteinase derived type II collagen neoepitope, CIIM--increased serum CIIM in subjects with severe radiographic osteoarthritis. *Clin Biochem.* 2011;44(5-6):423-429. PMID: 21223960.
2. Bayliss MT, Hutton S, Hayward J, Maciewicz RA. Distribution of aggrecanase (ADAMts 4/5) cleavage products in normal and osteoarthritic human articular cartilage: the influence of age, topography and zone of tissue. Osteoarthritis and Cartilage 2001, 9(6):553-560. PMID: 11520169.
3. Belcher C, Yaqub R, Fawthrop F, Bayliss M, Doherty M. Synovial fluid chondroitin and keratan sulphate epitopes, glycosaminoglycans, and hyaluronan in arthritic and normal knees. *Ann Rheum Dis.* 1997;56(5):299-307.
4. Bhosale AM, Kuiper JH, Johnson WE, Harrison PE, Richardson JB. Midterm to long-term longitudinal outcome of autologous chondrocyte implantation in the knee joint: a multilevel analysis. *Am J Sports Med.* 2009;37(suppl 1):131S-8S. PMID: 19861698.
5. Bondeson J, Wainwright SD, Lauder S, Amos N, Hughes CE. The role of synovial macrophages and macrophage-produced cytokines in driving aggrecanases, matrix metalloproteinases, and other destructive and inflammatory responses in osteoarthritis. Arthritis Res Ther. 2006;8(6):R187. PMID: 17177994.
6. Brittberg M, Lindahl A, Nilsson A, Ohlsson C, Isaksson O, Peterson L. Treatment of deep cartilage defects in the knee with autologous chondrocyte transplantation. *N Eng J Med.* 1994;331:889-895. PMID: 8078550.
7. Catterall JB, Stabler TV, Flannery CR, Kraus VB. Changes in serum and synovial fluid biomarkers after acute injury (NCT00332254). *Arthritis Res Ther.* 2010;12(6):R229. PMID: 21194441.

8. Chen P, Zhu S, Wang Y, Mu Q, Wu Y, Xia Q, Zhang X, Sun H, Tao J, Hu H, Lu P, Ouyang H. The amelioration of cartilage degeneration by ADAMTS-5 inhibitor delivered in a hyaluronic acid hydrogel. *Biomaterials.* 2014;35(9):2827-2836. PMID: 24424207.
9. Chockalingam PS, Sun W, Rivera-Bermudez MA, Zeng W, Dufield DR, Larsson S, Lohmander LS, Flannery CR, Glasson SS, Georgiadis KE, Morris EA. Elevated aggrecanase activity in a rat model of joint injury is attenuated by an aggrecanase specific inhibitor. *Osteoarthritis and Cartilage.* 2011;19(3):315-323. PMID: 21163358.
10. Cicuttini F, Ding C, Wluka A, Davis S, Ebeling PR, Jones G. Association of Cartilage Defects With Loss of Knee Cartilage in Healthy, Middle-Age Adults. A Prospective Study. *Arthritis and Rheumatism.* 2005;52:2033-2039. PMID: 15986359
11. Daghestani HN, Pieper CF, Kraus VB. Soluble macrophage biomarkers indicate inflammatory phenotypes in patients with knee osteoarthritis. *Arthritis Rheum*. 2007;67(4):956-963. PMID: 25544994.
12. Ding C, Cicuttini F, Jones G. Tibial subchondral bone size and knee cartilage defects: relevance to knee osteoarthritis. *Osteoarthritis and Cartilage*. 2007;15:479-486. PMID: 17291789.
13. Dugard MN, Kuiper JH, Parker J, Roberts S, Robinson E, Harrison PE, Roberts S, Richardson JB. Development of a tool to predict outcome of Autologous Chondrocyte Implantation. Cartilage. 2016 (In press).
14. Ehrich EW, Davies GM, Watson DJ, Bolognese JA, Seidenberg BC, Bellamy N. Minimal perceptible clinical improvement with the Western Ontario and McMaster Universities osteoarthritis index questionnaire and global assessments in patients with osteoarthritis. J Rheumatol. 2000;27(11):2635-41. PMID: 11093446.

15. Erben RG, Silva-Lima B, Reischl I, Steinhoff G, Tiedemann G, Dalemans W, Vos A, Janssen RT, Le Blanc K, van Osch GJ, Luyten FP. White Paper on How to Go Forward with Cell-Based Advanced Therapies in Europe. *Tissue Eng Part A.* 2014;20(19-20):2549-2554. PMID: 24749762.
16. Felson DT. The current and future status of biomarkers in osteoarthritis. *J Rheumatol.* 2014;41(5):834-836. PMID: 24788463.
17. Harris JD, Siston RA, Brophy RH, Lattermann C, Carey JL, Flanigan DC. Failures, re-operations, and complications after autologous chondrocyte implantation-a systematic review. *Osteoarthritis and Cartilage.* 2011;19(7):779-791. PMID: 21333744.
18. Germaschewski FM, Matheny CJ, Larkin J, Liu F, Thomas LR, Saunders JS, Sully K, Whittall C, Boyle Y, Peters G, Graham NM. Quantitation of ARGS aggrecan fragments in synovial fluid, serum and urine from osteoarthritis patients. *Osteoarthritis and Cartilage.* 2014;22(5):690-697. PMID: 24583346.
19. Jaiswal PK, Bentley G, Carrington RW, Skinner JA, Briggs TW. The adverse effect of elevated body mass index on outcome after autologous chondrocyte implantation. *J Bone Joint Surg (Br).* 2012;94(10):1377-1381. PMID: 23015564.
20. Jungmann PM, Salzmann GM, Schmal H, Pestka JM, Südkamp NP, Niemeyer P. Autologous chondrocyte implantation for treatment of cartilage defects of the knee: what predicts the need for reintervention? *Am J Sports Med.* 2012;40(1):58-67. PMID: 21969180.
21. Kraus VB, Stabler TV, Kong SY, Varju G, McDaniel G. Measurement of synovial fluid volume using urea. *Osteoarthritis and Cartilage.* 2007;15(10):1217-1220. PMID: 17507255.
22. Larsson S, Lohmander LS, Struglics A. An ARGS-aggrecan assay for analysis in blood and synovial fluid. *Osteoarthritis and Cartilage* 2014;22(2):242-249. PMID: 24361794.

23. Lohmander LS, Neame PJ, Sandy JD. The structure of aggrecan fragments in human synovial fluid. Evidence that aggrecanase mediates cartilage degradation in inflammatory joint disease, joint injury, and osteoarthritis. *Arthritis Rheum.* 1993;36(9):1214-1222. PMID: 8216415.
24. Lynn HS. Maximum likelihood inference for left-censored HIV RNA data. *Stat Med*. 2001;20(1):33-45. PMID: 11135346.
25. McCarthy HS, Roberts S. A histological comparison of the repair tissue formed when using either Chondrogide(®) or periosteum during autologous chondrocyte implantation. *Osteoarthritis Cartilage*. 2013;21(12):2048-57. PMID: 24161708.
26. Nair A, Kanda V, Bush-Joseph C, Verma N, Chubinskaya S, Mikecz K, Glant TT, Malfait AM, Crow MK, Spear GT, Finnegan A, Scanzello CR. Synovial fluid from patients with early osteoarthritis modulates fibroblast-like synoviocyte responses to toll-like receptor 4 and toll-like receptor 2 ligands via soluble CD14. *Arthritis Rheum.* 2012;64(7):2268-2277. PMID: 22492243.
27. Nganvongpanit K, Pothacharoen P, Chaochird P, Klunklin K, Warrit K, Settakorn J, Pattamapaspong N, Luevitoonvechkij S, Arpornchayanon O, Kongtawelert P, Pruksakorn D. Prospective evaluation of serum biomarker levels cartilage repair by autologous chondrocyte transplantation and subchondral drilling in a canine model. *Arth Res Ther.* 2009;11(3):R78. PMID: 19470154.
28. Nie L, Chu H, Liu C, Cole SR, Vexler A, Schisterman EF. Linear Regression with an Independent Variable Subject to a Detection Limit. *Epidemiology.* 2010;21(Suppl 4): S17–S24. PMID: 21422965.
29. Niemeyer P, Pestka JM, Kreuz PC, Erggelet C, Schmal H, Suedkamp NP, Steinwachs M. Characteristic complications after autologous chondrocyte implantation for cartilage defects of the knee joint. *Am J Sports Med.* 2008, 36(11):2091-2099. PMID: 18801942.

30. Niemeyer P, Pestka JM, Salzmann GM, Südkamp NP, Schmal H. Influence of cell quality on clinical outcome after autologous chondrocyte implantation. Am J Sports Med. 2012;40(3):556-61. PMID: 22174342.
31. Peterson L, Brittberg M, Kiviranta I, Akerlund EL, Lindahl A. Autologous chondrocyte transplantation. Biomechanics and long-term durability. *Am J Sports Med.* 2002;30(1):2-12. PMID: 11798989.
32. Richardson JB, Caterson, B, Evans EH, Ashton BA, Roberts S. Repair of human articular cartilage after implantation of autologous chondrocytes. *J Bone Joint Surg (Br).* 1999;81:1064-1068. PMID: 10615987.
33. Roberts S, Evans H, Wright K, van Niekerk L, Caterson B, Richardson JB, Kumar KH, Kuiper JH. ADAMTS-4 activity in synovial fluid as a biomarker of inflammation and effusion. *Osteoarthritis and Cartilage.* 2015;23(9):1622-1626. PMID: 26003949.
34. Roos EM, Lohmander LS. The Knee injury and Osteoarthritis Outcome Score (KOOS): from joint injury to osteoarthritis. Health Qual Life Outcomes. 2003;1:64. PMID: 14613558.
35. Sandy JD, Verscharen C. Analysis of aggrecan in human knee cartilage and synovial fluid indicates that aggrecanase (ADAMTS) activity is responsible for the catabolic turnover and loss of whole aggrecan whereas other protease activity is required for C-terminal processing in vivo. Biochem J 2001, 358:615-626. PMID: 11535123.
36. Sharif M, George E, Shepstone L, Knudson W, Thonar EJ, Cushnaghan J, Dieppe P. Serum hyaluronic acid level as a predictor of disease progression in osteoarthritis of the knee. *Arthritis Rheum.* 1995;38(6):760-767. PMID: 7779118.
37. Saris DB1, Vanlauwe J, Victor J, Almqvist KF, Verdonk R, Bellemans J, Luyten FP; TIG/ACT/01/2000&EXT Study Group. Treatment of symptomatic cartilage defects of the knee: characterized chondrocyte implantation results in better

clinical outcome at 36 months in a randomized trial compared to microfracture. Am J Sports Med;37 Suppl 1:10S-19S. PMID: 19846694.

38. Smith HJ, Richardson JB, Tennant A. Modification and validation of the Lysholm Knee Scale to assess articular cartilage damage. *Osteoarthritis and Cartilage.* 2009;17:53-58. PMID: 18556222.

39. Song RH, Tortorella MD, Malfait AM, Alston JT, Yang Z, Arner EC, Griggs DW. Aggrecan degradation in human articular cartilage explants is mediated by both ADAMTS-4 and ADAMTS-5. Arthritis Rheum 2007, 56(2):575-585.

40. Steyerberg E. Clinical prediction models: a practical approach to development, validation, and updating. New York (Springer) 2009.

41. Vanlauwe J, Saris DB, Victor J, et al. Five-year outcome of characterized chondrocyte implantation versus microfracture for symptomatic cartilage defects of the knee: early treatment matters. *Am J Sports Med.* 2011;39(12):2566-2574. PMID: 17265492.

42. Vasara AI, Konttinen YT, Peterson L, Lindahl A, Kiviranta I. Persisting high levels of synovial fluid markers after cartilage repair. Clin Orthop Relat Res. 2009;467:267-272. PMID: 18709427.

43. Wainwright SD, Bondeson J, Hughes CE. An alternative spliced transcript of ADAMTS4 is present in human synovium from OA patients. *Matrix Biol.* 2006;25(5):317-320. PMID: 16723216.

44. Wainwright SD, Bondeson J, Caterson B, Hughes CE. ADAMTS-4_v1 is a splice variant of ADAMTS-4 that is expressed as a protein in human synovium and cleaves aggrecan at the interglobular domain. *Arthritis Rheum.* 2013;65(11):2866-2875. PMID: 23897278.

45. Wasilko SM, Tourville TW, DeSarno MJ, Slauterbeck JR, Johnson RJ, Struglics A, Beynnon BD. Relationship between synovial fluid biomarkers of articular cartilage metabolism and the patient's perspective of outcome depends on the

severity of articular cartilage damage following ACL trauma. J Orthop Res. 2016;34(5):820-7. PMID: 26497486.

46. Zhang E1, Yan X, Zhang M, Chang X, Bai Z, He Y, Yuan Z. Aggrecanases in the human synovial fluid at different stages of osteoarthritis. *Clin Rheumatol.* 2013;32(6):797-803. PMID: 23370724.