**Identification of Candidate Synovial Fluid Biomarkers for the Prediction of Patient Outcome following Microfracture or Osteotomy**

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**Abstract**

Background: Biomarkers are needed to predict clinical outcome for microfracture and osteotomy surgeries to ensure patients can be better stratified to receive the most appropriate treatment.

Hypothesis/purpose: To identify novel biomarker candidates and to investigate the potential of a panel of protein biomarkers for the prediction of clinical outcome following treatment with microfracture or osteotomy.

Study Design: Descriptive Laboratory Study

Methods: Label-free quantitation following liquid chromatography-tandem mass-spectrometry of dynamic range compressed synovial fluids (SF) from individuals who responded excellently or poorly (based on the change in Lysholm score) to microfracture (n=6) or osteotomy (n=7) was used to identify novel candidate biomarker proteins. Biomarkers which were (a) identified in this proteomic analysis, (b) which relate to OA severity or (c) with predictive value in another early OA therapy ( autologous cell implantation) were measured in the SF of 19 and 13 patients prior to microfracture or osteotomy, respectively, using commercial immunoassays and normalised to urea. These were aggrecanase-1 (ADAMTS-4), cartilage oligomeric matrix protein (COMP), hyaluronan (HA), Lymphatic Vessel Endothelial Hyaluronan Receptor-1 (LYVE-1), matrix metalloproteinase (MMP)-1 and -3, soluble CD14 (sCD14), S100 calcium binding protein A13 (S100A13) and 14-3-3 protein theta (YWHAQ). Levels of COMP and HA were also measured in the plasma of these patients. To find predictors of postoperative function multivariable regression analyses were performed.

Results: Proteomic analyses highlighted YWHAQ and LYVE-1 as being differentially abundant between the clinical responders/improvers and non-responders following microfracture. A linear regression model following backward variable selection could relate pre-operative concentrations of SF proteins (HA, YWHAQ, LYVE-1), activity of ADAMTS-4 and patient demographics (smoker status and gender) with 12 month post-microfracture Lysholm score. Further a generalized linear model with elastic net penalisation indicated that lower pre-operative activity of ADAMTS-4 in SF, along with being a non-smoked and younger at the time of operation were indicative of a higher post-operative Lysholm score (improved joint function) following osteotomy surgery.

Conclusion: We have identified biomarkers and generated regression models with the potential to predict clinical outcome in patients treated with microfracture or osteotomy of the knee.

What is known about the subject: There are no published studies, to our knowledge that have aimed to identify biomarkers that can predict the outcome to the routinely used surgeries in the knee, microfracture and osteotomy.

What this study adds to existing knowledge: We have identified proteins and patient demographics that could be used to help predict which surgical intervention may be most appropriate for an individual. Further our proteomic studies have highlighted a number of other candidate predictive biomarkers which provide a resource for further investigation.

**Key Words**

Early osteoarthritis, knee, microfracture, osteotomy, predictive biomarkers, synovial fluid, proteomics, immunoassays, enzyme activity assays

**Background**

Some of the most widely used surgical approaches that aim to repair chondral/osteochondral defects of the knee include microfracture and osteotomy 10,40. Despite advanced cell therapy approaches, such as Autologous Chondrocyte Implantation (ACI) having been highlighted as more efficacious and cost-effective for repair of chondral/osteochondral defects of the knee 26, these surgeries are not available in non-specialist orthopaedic centres and they are more expensive to conduct; hence alternative surgeries such as microfracture and osteotomy are often used. These surgeries can be economical treatments, but only for those individuals in whom they are successful; therefore, there is a need for early identification of patients who are likely to benefit from them. The Osteoarthritis Research Society International (OARSI) have previously highlighted the need to identify biomarkers that can predict which patients are most likely to benefit from surgical interventions to delay or prevent the development of osteoarthritis (OA)16 and we are unaware of any published studies that have aimed at addressing this need for microfracture or osteotomy.

Work to identify biomarkers (biochemical and imaging) for the diagnosis and prognosis of OA has been numerous and on-going for many years13. However, far fewer studies have focused upon the identification of biomarkers for prediction of clinical outcome in response to a surgical intervention to delay the onset or to prevent OA. To our knowledge, the only published studies that have used human samples with this aim have been related to ACI 11,12,45 or Anterior Cruciate Ligament (ACL) reconstrution18. Wright et al. (2017) demonstrated that assessment of proteins of known biological relevance to OA within the synovial fluid (SF) can identify protein markers that when combined with known demographic/surgical risk factors can be used to predict the success of surgical treatment with ACI 45. Further in support of this approach, Latterman et al. (2018) were able to demonstrate that assessment of OA related proteins, as well as inflammatory cytokines, in SF collected at the time of surgery could be used to differentiate between individuals who clinically improved and those who did not at two years following ACL reconstruction18. Alternative to assessing OA- related proteins, the use of unbiased discovery based approaches have been employed to identify within-treatment proteome shifts that are unique to ACI non-responders 11,12. In the study described here we employed both these targeted and non-targeted approaches. We hypothesise that these approaches can be used to identify protein biomarkers that may predict an individual’s probability of a successful outcome in response to microfracture or osteotomy.

Proteins were selected for our targeted analysis that have known biological relevance to cartilage degeneration/turnover and OA, along with proteins that have previously been highlighted as candidate biomarkers for the prediction of outcome to ACI via non-targeted proteomic analyses11,12. The cartilage matrix related proteins and enzymes assessed in this study were cartilage oligomeric matrix protein (COMP), hyaluronan (HA), A disintegrin and metalloproteinase with thrombospondin motifs 4 (ADAMTS-4; Aggrecanase-1) and matrix metalloproteinases (MMP) -1 and -3. COMP is a non-collagenous glycoprotein involved in collagen-collagen interactions43. HA is a glycosaminoglycan which constitutes a key component of the cartilage extracellular matrix36. Both COMP and HA have long been suggested as prognositic biomarkers of OA progression36,37. ADAMTS-4, MMP-1 and MMP-3 are all key enzymes in regulating cartilage homeostasis. ADAMTS-4 cleaves large chondroitin sulphate hyaluronan-binding proteoglycans including aggrecan33. MMPs also breakdown other key cartilage matrix components, specifically collagen II by MMP-1 and a broad range of matrix components including aggrecan by MMP-32425. soluble CD14 (sCD14) was assessed as this protein has been associated with OA progression and pain8. CD14 is found on the surface of monocytes and macrophages and regulates the production of a number of inflammatory mediators8. S100 calcium binding protein A13 (S100A13) was also assessed; this protein having previously been highlighted as having potential to predict ACI outcome following untargeted proteomic analysis12. S100A13 is a member of the S100 family of proteins which have a wide variety of extracellular functions, many of which act as alarmins which contribute to the regulation of immune and inflammatory responses and post-traumatic injury respones46 , however no specific relation between S100A13 and the joint environment homeostasis/inflammatory response has been reported.

Within the field of OA, much of the work aimed at identifying prognostic and predictive biomarkers has relied on SF, as this fluid represents the whole joint environment. However, identification of protein biomarkers that can be assessed within the blood rather than the SF remains the ultimate goal in clinical practice as this would provide a less invasive method of predicting which individuals are likely to benefit from these surgical procedures to treat cartilage defects. Therefore, we have also assessed some commonly studied OA-related biomarker proteins within the plasma of osteotomy and microfracture patients.

**Methods**

**Patients**

Following local research ethical committee approval (11/NW/0875 and 06/Q6201/9; see declarations) and with informed consent blood and SF samples were collected from individuals undergoing osteotomy or microfracture at our centre between 2015 and 2019, along with samples collected in our research group’s biobank since 2006. Prior to surgery, patients’ demographic details were recorded and their functional status was determined via completion of the modified Lysholm patient-reported outcome measure 22,38. The modified Lysholm score ranges from 0 to 100, with 100 representing ‘perfect’ knee function22. Individuals were deemed to respond to surgery if they demonstrated a minimally clinically important difference (MCID) of a 10 point increase in the Lysholm score between pre- and post-operative scores 9,32,35. Post-operative scores were collected at approximately 12 months following surgery (13±6.5 months (median±IQR)). The level of severity of a patient’s OA pre-surgery was determined using Kellgren-Lawrence grading of radiographs14 independently by two consultant orthopaedic surgeons. A mean of the two surgeons scores was taken as the pre-operative Kellgren-Lawrence score. The details of these patients are described in Table 1A. Samples from the whole patient cohort (Table 1A) were assessed for all biomarker proteins including those related to OA and cartilage and to validate those proteins identified in this study via exploratory proteomic analysis.

A group of samples were selected for proteomic analysis from a sub-group of patients who demonstrated the greatest improvement in clinical score (responders) or least improvement/worsening of function (non-responders) at 12 (+/- 2) months following osteotomy or microfracture. Other selection criteria for the proteomic study included: having > 2ml of SF sample for analysis, no blood contamination staining of the SF, as this has been demonstrated to alter the detection of proteins within the fluid 44 and a dilution factor of <14 (detailed in Table 2).

**Table 1.** Demographic data for patient participants treated with either osteotomy or microfracture

|  |  |  |  |
| --- | --- | --- | --- |
|  | **Microfracture** | **Osteotomy** | |
| **(n=19)** | **(n=13)** | |
| **A** | | | |
| Age, years (median (IQR)) [range] | 34 (22) [17-67] | 46(9) [31-58] | |
| Male (n) | 12 | 8 | |
| BMI, kg/m2 (median (IQR)) [range] | 25(5) [19-36] | 30(2) [21-34] | |
| Baseline Lysholm Score (median (IQR)) [range] | 50 (30) [23-88] | 52(17) [17-66] | |
| Post-operative Lysholm Score (median (IQR)) [range] | 54 (35) [16-96] | 79(33) [38-92] | |
| Baseline Kellgren-Lawrence Score (median (IQR)) [range] | 0.5(1) [0-2] | 2(1) [1-3] | |
| Treatment side- Right Leg | 9 | 6 | |
| Treatment side- Left leg | 10 | 7 | |
| Smokers (n) | 1 | 2 | |
| **B** | | | |
| **Synovial Fluid Markers (median (IQR)) [range]** | | | |
| COMP (mgml-1) | 157.3(90.3) [29.1-383.2] | 147.1(129.8) [44.5-310.6] | |
| HA (mgml-1) | 17.7(9.1) [3.1-34.0] | 14.2(23.1) [1.6-48.9] | |
| sCD14 (ngml-1) | 765.6(1516.9) [11.3-5014.7] | 2308.4(2144.2) [479.1-3880.2] | |
| ADAMTS-4/aggrecanase-1 (ngml-1) | 0.4(21.4) [0.4-44.0] | 0.4(17.4) [0.4-34] | |
| MMP-1 (ngml-1) | 0.3(5.3) [0.04-22.9] | 1.7(7.9) [0.3-10.1] | |
| MMP-3 (ngml-1) | 136.2(205.2) [0-5060.9] | 532.7(515.8) [163.8- 2867.8] | |
| S100A13 (pgml-1) | 2529.4(2116.3) [946.5-6795.6] | 1552.5(2723.2) [0-5807.8] | |
| YWHAQ (ngml-1) | 0.5(0.5) [0.5-10.6] | 1.5(2.0) [0.5-7.98] | |
| LYVE-1 (ngml-1) | 4.1(4.1) [1.5-16.1] | 1.1(1.5) [0.5-5.3] | |
| **Plasma Markers (median(IQR)) [range]** | | |
| COMP (ng/ml) | 491.4(155.3) [342.4-758.2] | 629.5(230.7) [342.4-1212.0] | |
| HA (ng/ml) | 0.5(5.0) [0.5-20.9] | 0.5(16.2) [0.5-57.8] | |

*Abbreviations: ADAMTS-4 (A disintegrin and metalloproteinase with thrombospondin motifs 4); BMI( body mass index); COMP (cartilage oligomeric matrix protein); HA (hyaluronan); IQR (interquartile range); LYVE-1 (Lymphatic Vessel Endothelial Hyaluronan Receptor-1); sCD14 (soluble CD14); S100A13 (S100 calcium binding protein A13); YWHAQ (14-3-3 protein theta).*

**Table 2.** Demographic data for patient participants whose synovial fluid samples were used for proteomic analysis and who responded clinically (responders) or who did not respond (non-responders) to either osteotomy or microfracture.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
|  | **Osteotomy** | | | **Microfracture** | | |
| **Responders (R) (n=3)** | **Non-responders (NR) (n=4)** | **p value**  **R v NR** | **Responders (R) (n=3)** | **Non-responders (NR)**  **(n=3)** | **p value**  **R v NR** |
| Difference in Lysholm score | 44 (21-62) | -8 (0 - -16) | 0.004 | 36 (16-72) | -3 (0 - -4) | 0.070 |
| Baseline Kellgren-Lawrence score | 2 (1.5-2.5) | 2.5 (1-3) | 0.465 | 0 (0-0) | 1 (1-2) | 0.070 |
| Age, yrs | 44 (29-59) | 40 (31-46) | 0.807 | 25 (19-34) | 38 (24-46) | 0.201 |
| Male (n) | 3 | 2 | >0.999 | 1 | 2 | >0.999 |
| BMI ((kg/m2) | 34 (30-35) | 30 (28-30) | 0.053 | 20 (19-21) | 34 (29-38) | 0.095 |
| Treatment side- right | 1 | 2 | >0.999 | 2 | 3 | >0.999 |
| Treatment side- left | 2 | 2 | >0.999 | 1 | 0 | >0.999 |
| Smoker (n) | 0 | 1 | >0.999 | 1 | 0 | 0.400 |
| Dilution factor of SF | 4 (0-9) | 7 (3-13) | 0.856 | 7 (6-8) | 7 (6-10) | 0.877 |

*None of the demographic parameters, other than difference in Lysholm scores, showed differences between responders (R) and non-responders (NR) in individuals whose synovial fluids (SFs) were compared for each surgical procedure (Lysholm, age, BMI, dilution factor: unpaired t-test; gender, treatment side, smoker status: Mann-whitney U test). Data are shown as median (range) unless otherwise indicated. Abbreviations: BMI (body mass index); SF (synovial fluid).*

**Synovial fluid and Plasma collection and storage**

SF was collected from patients’ knee joints immediately prior to microfracture or osteotomy surgery, by injecting 20 ml of saline and then extending and flexing the leg at least 20 times prior to intra-articular aspiration of as much SF as possible31. At this time, blood samples were also collected by venepuncture. SF and plasma were then centrifuged at 6,000 *g* for 15 mins at 4˚C and the supernatant removed before being divided into aliquots and stored in -196˚C liquid nitrogen prior to analyses.

**Proteomic Analysis of Synovial Fluids**

Sample preparation and analysis using proteomics

SF samples were not pooled at any point of the proteomic sample preparation or mass spectrometry stages, therefore the protein abundance was quantified for each of the thirteen samples and mean protein abundance for each experimental group was calculated prior to the analysis of protein changes.

SF Preparation and protein equalisation using ProteoMinerTM

ProteoMinerTM beads (BioRad, Hemel Hempstead, UK) were used to compress the dynamic range of proteins to allow improved identification of low abundance proteins as described previously 12,28. The total protein concentration of hyaluronidase (1 mg/ml) treated SFs12 was quantitated using a PierceTM 660 nm protein assay (Thermo Scientific, Hemel Hempstead, UK). Five mg of total protein was incubated with ProteoMinerTM beads following kit instructions. Proteins attached to the beads were then treated with 0.05% (w/v) RapiGest (Waters, Manchester, UK) in 25 mM ammonium bicarbonate for 10 min at 80°C prior to reduction, alkylation and *in situ* protein digestion which was carried out using trypsin in LoBind protein tubes (Eppendorf, Stevenage, UK)) without removal of the beads. Samples were acidified using trifluoroacetic acid to a final concentration of 0.5% (v/v) and multiple centrifugation steps to inactivate and precipitate the RapiGest detergent. Peptide-containing supernatant fractions were then frozen at -20˚C prior to liquid chromatography- tandem mass spectrometry (LC-MS/MS).

Liquid chromatography- tandem mass spectrometry ass-spectrometry (LC-MS/MS) and label-free quantification

A NanoAcquityTM ultraperformance LC (Waters, Manchester, UK) coupled online to a Q-Exactive Quadrupole-Orbitrap instrument (Thermo-Fisher Scientific Hemel Hempstead, UK) was used to analyse tryptic peptides on a 2h gradient 12,28. For label-free quantification, raw files of the acquired data were analysed (as described previously12), in ProgenesisQITM software (Waters, Manchester, UK) 27 and the top five spectra for each feature were used for peptide identification in a locally implemented Mascot server (Version 2.3.01), searching against the Unihuman Reviewed database. Peptide matches above an identify threshold were adjusted to give a false discovery rate (FDR) of 1% before the protein identifications were re-imported into ProgenesisQITM for the label-free relative quantification of unique peptides. Statistical analysis was performed using ProgenesisQITM software; briefly, transformed normalised abundances were used for one-way analysis of variance (ANOVA) and all peptides (with p<0.05) of an identified protein were included. In order to select proteins for biochemical validation, the mean abundance of each protein in each of the experimental groups was calculated and significant proteins (false discovery rate; p<0.05) with a ≥±2.0 fold change (FC) between comparator groups reported.

**Assessment of protein abundances using Enzyme-Linked Immunosorbent Assay (ELISA)**

Assessment of proteins identified in the LC-MS/MS proteomic analyses using ELISA

14-3-3 Protein Theta (YWHAQ) was selected as a protein to validate the LC-MS/MS findings as this protein was only detected in non-responders and not detected at all in responders to microfracture, deeming it an ideal candidate biomarker. Lymphatic Vessel Endothelial Hyaluronan Receptor-1 (LYVE-1)was also selected to validate the LC-MS/MS findings because it can bind to hyaluronan 47, a key component of articular cartilage and SF. LYVE-1 is also highly expressed in the synovium of OA

patients who have synovial villous hypertrophy and chronic inflammatory cell infiltrate 47. YWHAQ and LYVE-1 were quantified using ELISAs (YWHAQ: Cusabio, Texas, USA; LYVE-1: R&D systems, **Wiesbaden**, Germany) according to the manufacturers’ instructions.SF from the same patients as were assessed in the LC-MS/MS analysis were used to validate the proteomic findings. These samples were assessed in duplicate and mean optical density values were used to calculate the protein concentration. SF was diluted 1:50 for the assessment of LYVE-1 and was assayed neat (i.e. not further diluted above the dilution due to lavage) for YWHAQ.The concentration of each protein was normalised to total protein concentration, to validate the LC-MS/MS, as equal concentrations of protein were loaded onto the ProteoMinerTM beads*.* Statistical analysis was performed in GraphPad Prism version 8.0. These proteins were then included in ‘targeted’ biomarker analyses in the larger cohort of patients (Table 1) and included in the multivariable regression modelling to determine whether they had any predictive value over and above other proteins.

Assessment of osteoarthritis and cartilage associated proteins using ELISA

Targeted analyses of proteins that are associated with the development of OA and with known cartilage biology were performed using ELISA. These included proteins which we have previously highlighted as associated with clinical outcome in patients undergoing cell therapy via ACI for the treatment of early OA 11,12,45. Levels of cartilage oligomeric matrix proteins (COMP) and HA in both the SF and plasma were assessed using an ELISA (BioVendor Laboratory Medicine, Czech Republic) and an enzyme-linked protein binding assay (Corgenix, CO), respectively, as described previously 45. As in our previous work, SF concentrations of soluble CD14 (sCD14) were measured using a Quantikine ELISA kit (Biotechne, MN) and ADAMTS-4 (Aggrecanase-1) enzyme activity assessed using an endpoint fluorometric substrate assay (SensoLyte 520 Aggrecanase-1 Assay Kit; AnaSpec, CA) 45. Concentrations of matrix metalloproteinases (MMP) -1 and -3 and S100 calcium binding protein A13 (S100A13) were assayed using duo-sets (MMP-1 & S100A13; Biotechne, MN) or Quantikine ELISAs (MMP-3; Biotechne, MN) 11,12. All assays were optimised to determine the appropriate sample dilution factor (over and above dilution due to lavage) as follows: ADAMTS-4- undiluted; sCD14 - 1:200; COMP- 1:1000; HA -1:3000; MMP-1 - 1:3; MMP-3 - 1:100; S100A13 1:20. Plasma samples were diluted 1:50 for COMP and assayed neat for HA. The concentration of each protein was normalised to the dilution factor of the SF. To determine the dilution of the SF (due to lavage), urea concentrations in the SF and plasma were assessed using a QuantiChrom urea assay kit (Universal Biologicals) and SF biomarker values were normalised to the urea concentration in blood plasma as described previously 17. The principal of this method to determine dilution factor is based on the fact that there is a strong correlation between the concentration of urea in the SF and plasma of an individual, therefore the SF volume can be calculated by assessing matched patient SF and plasma samples17 . Median baseline protein biomarker levels are summarised in Table 1B.

**Statistical Analyses**

Data analyses were perfomed using the statistical programming language R version 4.0.2 (2020-6-22) 41. Independent models were built for both the total microfracture (n=19) and osteotomy (n=13) cohorts. The post-operative Lysholm score was used as the dependent variable and independent variables were pre-operative Lysholm score, patient age at the time of microfracture or osteotomy, body mass index (BMI), smoker status and baseline level of the nine SF biomarkers (COMP, HA, sCD14, ADAMTS-4, MMP-1, MMP-3, S100A13, LYVE-1 and YWHAQ) and the two plasma biomarkers (HA and COMP). Imputed values were used when there were non-detects for any of the proteins assessed using ELISA 45. For sCD14, plasma HA and YWHAQ the imputed value was taken as equal to (1/√2) times the lowest detected value (sCD14: 11.3; plasma HA: 0.5; YWHAQ: 0.5), which was the value applied to ADAMTS-4 non-detects in our previous study (0.40) 45. Any missing data were imputed using multiple imputation by chained equations1. The number of model features compared to the number of observations was relatively high and therefore linear regression with elastic net penalization was performed. Elastic net penalization is a combination of least absolute shrinkage and selection operator (LASSO) and ridge regression. LASSO penalization shrinks each predictor differently and allows variables to be removed entirely by shrinking coefficients to 0 42,50, whereas for ridge regression the penalty term shrinks the effect of the predictor equally and none are reduced to 0. Thereby, elastic net can eliminate features entirely and allows for the reduction of impact of less important model features.

To provide confidence in the findings and to build models which best represented the different datasets, multivariable linear regression models were also built and variables selected using backward variable selection. Backwards elimination removes the least significant effect that doesn’t meet the level for remaining in the model and this is repeated until no other effect in the model meets the specified level for removal. This method allows for co-linearity between variables to be accounted for6.

Protein markers that demonstrated a significant contribution to the models (*P* <0.05) were then assessed for their potential to predict outcome to surgery based on the ‘responder’ definitions given above, specifically a MCID in Lysholm score of at least 10 points at 12 months post-surgery. When categorised as ‘responders’ and ‘non-responders’ there were a small number of patients in each response arm for the two different surgeries. Therefore to assess if there were differences in the activity/expression of these enzymes/proteins, Mann-Whitney tests were performed using GraphPad Prism version 8.0.

**Results**

**Patients**

Thirteen patients were selected, from the overall cohort, for proteomic analysis of the SF, based on the selection criteria detailed within the methods. Three donors were considered extreme responders to osteotomy with a mean improvement of 44 Lysholm points (range 21-62) and four donors were considered non-responders with a mean decrease in Lysholm score of 8 points (range 0-16). Following microfracture, three donors were deemed extreme responders (mean improvement 26 Lysholm points (range 16-72)) and a further three donors were deemed extreme non-responders (mean decrease of 3 Lysholm points (range 0-4)). The demographic information for these patients is shown in Table 2.

To assess candidate biomarkers in a larger cohort, 13 patients undergoing osteotomy and 19 patients undergoing microfracture were included in this study (Table 1). The mean baseline Lysholm score was 49.0(±13.9), which improved to 72.0(±21.0) at 16(±8.9) months following osteotomy surgery, whereas for microfracture patients the mean baseline Lysholm score was 50.7(±18.7), which only improved to 58.7(±25.6) at 15.2(±9.6) months post-operatively. When defined as responders or non-responders to surgery based on an MCID of 10 Lysholm points, 8 individuals were responders (37(16-62)(median(range)) increase in Lysholm points) and 5 were non-responders (-4(0- -16) (median(range)) Lysholm point change) to osteotomy and 8 patients were responders (23(12-72) (median(range)) Lysholm point increase) and 11 non-responders (-3(8- -34)(median(range)) Lysholm point change) to microfracture surgery. This study comprised of samples from 20 males (8 osteotomy and 12 microfracture) and 12 females, aged between 17 and 67 years at the time of surgery. Table 1A details the demographic variables of these patients.

**Differential abundance of synovial fluid proteins in non-responders compared to responders to Microfracture identified using proteomic analysis**

Individuals who did not respond well clinically to microfracture demonstrated a differential baseline proteome compared to those who responded well. Thirty proteins were differentially abundant (±2.0 fold; p<0.05) in the pre-operative SF of responders compared to non-responders of microfracture (Table 3). 14-3-3 protein theta (YWHAQ) was only present in the SF of the selected individuals that did not respond to microfracture and no presence of this protein was detected using LC-MS/MS in the responders. Conversely, small ubiquitin-related modifier 4 (SUMO4) was only detected in SF of responders to microfracture and not in the non-responders. Another of these proteins which was of biological interest because it can bind to hyaluronan 47, LYVE-1, was six-fold higher in the selected microfracture responders compared to non-responders.

**Differential abundance of synovial fluid proteins in non-responders compared to responders to Osteotomy identified using proteomic analysis**

A SF proteome shift was found between individuals who did or did not respond well to osteotomy. Table 4 demonstrates the 15 proteins that were differentially abundant (±2.0 fold; p<0.05) in the SF between the two different clinical outcome groups. Both YWHAQ and an undetectable protein KIAA1007 demonstrated a much higher abundance in the non-responders to osteotomy compared to the responders, with YWHAQ again being undetected in the responders using LC-MS/MS.

**Biochemical Validation of Proteomic Analyses**

14-3-3 Protein Theta (YWHAQ) was confirmed to have undetectable concentrations in microfracture responders and detectable concentrations only in microfracture non-responders when measured using ELISA (Figure 1). We also assessed YWHAQ using ELISA in the osteotomy samples used for the proteomic analyses but found that for two of the responder samples detectable levels were measured using ELISA, whereas they were not detected at all via LC-MS/MS (data not shown).

**Table 3:** Fold-change of proteins that are differentially abundant (±2.0 fold; false discovery rate p<0.05) in the pre-operative synovial fluid of individuals that do not improve following microfracture (non-responders; n=3) compared to those that improve (responders; n=3).

|  |  |  |
| --- | --- | --- |
| **Protein** | | **Fold Change** |
| **Description** | **Accession** |
| Small ubiquitin-related modifier 4 (SUM04) | Q6EEV6 | Infinity |
| 55 kDa erythrocyte membrane protein | Q00013 | -2103005.6 |
| Protein argonaute-1 | Q9UL18 | -147.4 |
| Complement C1r subcomponent-like protein | Q9NZP8 | -11.0 |
| Lymphatic vessel endothelial hyaluronic acid receptor 1 (LYVE-1) | Q9Y5Y7 | -6.0 |
| Peroxiredoxin-4 | Q13162 | -4.4 |
| Adiponectin | Q15848 | -2.8 |
| IgGFc-binding protein | Q9Y6R7 | -2.7 |
| Ubiquitin-conjugating enzyme E2 variant 1 | Q13404 | -2.5 |
| Alpha-2-macroglobulin | P01023 | -2.4 |
| Lysosomal acid phosphatase | P11117 | -2.1 |
| Alpha-lactalbumin | P00709 | 2.0 |
| Coagulation factor XI | P03951 | 2.1 |
| Beta-Ala-His dipeptidase | Q96KN2 | 2.2 |
| Ficolin-3 | O75636 | 2.5 |
| Retinol-binding protein 4 | P02753 | 2.5 |
| Serine protease HTRA1 | Q92743 | 2.6 |
| 28S ribosomal protein S34, mitochondrial | P82930 | 2.9 |
| Peptidyl-glycine alpha-amidating monooxygenase | P19021 | 3.1 |
| Dickkopf-related protein 3 | Q9UBP4 | 3.1 |
| Serum amyloid P-component | P02743 | 3.1 |
| Ribonuclease 4 | P34096 | 3.3 |
| Insulin-like growth factor II | P01344 | 3.6 |
| eIF-2-alpha kinase activator GCN1 | Q92616 | 5.2 |
| Glycosylphosphatidylinositol anchor attachment 1 protein | O43292 | 5.4 |
| Mannan-binding lectin serine protease 2 | O00187 | 6.8 |
| Poly(rC)-binding protein 3 | P57721 | 10.7 |
| Sodium channel protein type 8 subunit alpha | Q9UQD0 | 11.8 |
| Platelet factor 4 | P02776 | 44.4 |
| 14-3-3 protein theta (YWHAQ) | P27348 | Infinity |

*Positive numbers denote an increase in the protein in non-responders; negative numbers denote an increase in the protein in responders.*

**Table 4:** Fold-change of proteins that are differentially abundant (±2.0 fold; false discovery rate p<0.05) in the pre-operative synovial fluid of individuals that do not improve following osteotomy (non-responders; n=4) compared to those that improve (responders; n=3)

|  |  |  |
| --- | --- | --- |
| **Protein** | | **Fold Change** |
| **Description** | **Accession** |
| Integrin alpha-M | P11215 | -5.0 |
| Cubilin | O60494 | -4.6 |
| AT-rich interactive domain-containing protein 5B | Q14865 | -4.3 |
| Filamin-A | P21333 | -4.1 |
| Cystatin-C | P01034 | -3.9 |
| Immunoglobulin kappa variable 3D-20 | A0A0C4DH25 | -2.9 |
| Ubiquitin-conjugating enzyme E2 variant 1 | Q13404 | -2.8 |
| Immunoglobulin heavy constant alpha 1 | P01876 | -2.7 |
| Serum amyloid A-2 protein | P0DJI9 | -2.3 |
| Immunoglobulin kappa variable 1-6 | A0A0C4DH72 | -2.1 |
| Hyaluronan and proteoglycan link protein 1 | P10915 | 2.2 |
| Insulin-like growth factor-binding protein 5 | P24593 | 7.3 |
| von Willebrand factor | P04275 | 9.7 |
| Uncharacterized protein KIAA1107 | Q9UPP5 | 263.0 |
| 14-3-3 protein theta (YWHAQ) | P27348 | Infinity |

*Positive numbers denote an increase in the protein in non-responders; negative numbers denote an increase in the protein in responders.*

**A**

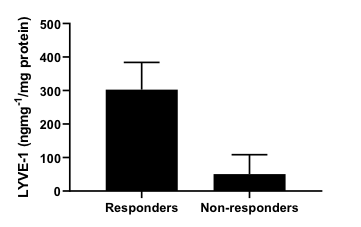


**D**

**B**

**C**

**E**



|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | **LC-MS/MS** | | **ELISA** | |
| **Fold-change** | **ANOVA (p-value)** | **Fold-change** | **t-test (p-value)** |
| **YWHAQ** | infinity | 0.0003 | Infinity | 0.0005 |
| **LYLVE-1** | 5.98 | 0.03 | 3.82 | 0.01 |

f

**Figure 1.** Two proteins, 14-3-3 protein theta (YWHAQ) and Lymphatic Vessel Endothelial Hyaluronan Receptor-1 (LYVE-1), that were identified by proteomic analysis as differentially abundant in the SF of non-responders compared to responders of microfracture were validated by ELISA. (A) Table demonstrating fold changes and p-values of differences between responders (n=3) cf. non-

responders (n=3) to microfracture. YWHAQ was detectable in the SF in non-responders (n=3) to microfracture but not in the SF of responders (n=3) as demonstrated by (B) label-free mass-spectrometry and (C) validated using quantitative ELISA. LYVE-1 was increased in abundance in the SF of responders (n=3) cf. non-responders (n=3) to microfracture as demonstrated using B) label-free mass-spectrometry and (C) validated using quantitative ELISA. Data are mean±SD.

Further the proteomic finding that LYVE-1 demonstrates greater abundance in microfracture responders compared to non-responders could be confirmed using biochemical analyses (responders: 154.6(45.0) pgml-1/ mg protein; non-responders: 40.4(14.6) pgml-1/ mg protein

(mean(SD)); p=0.01) (Figure 1). These proteins were therefore included in further studies to assess

their predictive potential compared to other biomarkers and demographic data in a larger patient cohort.

**Biomarker levels**

SF and plasma samples were included from all 32 patients for analysis of targeted OA-related biomarkers and additionally, candidate markers identified in our non-targeted proteomic study. The mean(±SEM) SF dilution factor was 7.8(±7.7) with the mean dilution factor being 5.3(±4.6) and 5.6(±4.5) in osteotomy responders and non-responders and 9.3(±6.9) and 8.4(±10.2) in microfracture responders and non-responders, respectively. Three SF proteins (sCD14, ADAMTS-4 and YWHAQ) and HA in plasma had samples with undetectable levels and values were imputed as described in the methods.

**Multivariable Linear Models of predictors of post-operative Lysholm score following surgical treatment**

**Multivariable Linear Models for Microfracture Outcome**

Generalized linear models (GLM) with elastic net penalization was built of the post-operative Lysholm following microfracture (Table 5). For the GLM of microfracture post-operative Lysholm score the r2 was low (0.5) and the RMSE high (26.4). Therefore, a linear regression model was generated following backward variable selection; this model better related the selected variables with the dependent variable, post-operative Lysholm score (r2 =0.7 RMSE= 13.6) (Table 6). There were a number of model parameters that significantly contributed to the linear regression model for microfracture outcomes, which were baseline levels of SF HA, YWHAQ, plasma HA, ADAMTS-4, LYVE-1 and patient smoker status and gender (Table 6). It is of note that despite the r2 being low for the generalized linear model with elastic net penalization for the post-microfracture Lysholm score, HA

**Table 5:** A generalized linear regression model with elastic net penalization for Predictors of the Post-Microfracture Lysholm Score

|  |  |  |  |
| --- | --- | --- | --- |
| **Microfracture (n=19)** | | | |
| **Component** | ***R2*  Value** | **RMSE** | **Variable Importance** |
| Total Model | .519 | 26.4 |  |
| HA (mgml-1) |  | | 100 |
| Baseline Lysholm | 28 |
| ADAMTS-4 (ngml-1) | 15 |

Final elastic net model parameters were alpha- 1, lambda- 7.82. Alpha is a value between 0 and 1, where 0 is pure ridge regression, 1 is pure LASSO and values between are a mixture of both. Lambda is the shrinkage factor applied to model coefficients

*Abbreviations: ADAMTS-4 (A disintegrin and metalloproteinase with thrombospondin motifs 4), HA (hyaluronan), RMSE (*Root mean square error)

**Table 6:** A linear regression model following backward variable selection for Predictors of the Post-Microfracture Lysholm Score

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Microfracture (n=19)** | | | | |
| **Component** | **Regression Coefficient (SEM)** | ***Adj. R2*  Value** | ***RMSE*** | ***P* Value** |
| Total Model |  | .71 | 13.6 | 0.04 |
| SF- HA (mgml-1) | -3.0(0.6) |  | | 0.003 |
| YWHAQ (ngml-1) | -8.09(1.7) | 0.003 |
| Plasma- HA (ngml-1) | -3.9(1.1) | 0.01 |
| Smoker | -49.9(14.6) | 0.01 |
| Gender | -27.3(8.0) | 0.02 |
| ADAMTS-4 (ngml-1) | 1.0(0.35) | 0.03 |
| LYVE-1 (ngml-1) | 0.001(0.0) | 0.04 |
| Baseline Lysholm | -0.81(0.3) | 0.05 |
| Kellgren-Lawrence | 17.5(7.3) | 0.05 |
| sCD14 (ngml-1) | 0.01(0.0) | 0.09 |
| MMP-3 (ngml-1) | 0.01(0.0) | 0.17 |
| BMI | -0.66(0.7) | 0.38 |
| MMP-1 (ngml-1) | -0.71(0.8) | 0.41 |

*Abbreviations: ADAMTS-4 (A disintegrin and metalloproteinase with thrombospondin motifs 4), BMI (Body Mass Index), HA (hyaluronan), LYVE-1 (Lymphatic Vessel Endothelial Hyaluronan Receptor-1 ), MMP-1 (matrix metalloproteinase-1), MMP-3 (matrix metalloproteinase-3), sCD14 (soluble CD14), RMSE (Root mean square error), YWHAQ (14-3-3 protein theta)*

was the variable which most strongly contributed to the models prediction value, again highlighting this cartilage matrix protein as a predictor of clinical outcome following microfracture.

**Multivariable Linear Models for Osteotomy Outcome**

A GLM with elastic net penalization was built of the post-operative Lysholm following osteotomy (Table 7) which had a strong correlation coefficient (r2=0.77) and low RMSE (12.1). The generalized linear model with elastic net penalization of post-operative Lysholm following osteotomy determined that the variables which most strongly contributed to the model were

smoker status, age at time of operation and pre-operative ADAMTS-4 activity (Table 6). These findings indicate that being a non-smoker, alongside being a younger age at the time of operation are related to an increased Lysholm score following osteotomy surgery.

**Assessment of candidate predictors in relation to a 10-point improvement in the Lysholm score following surgical treatment**

The biomarker proteins which significantly contributed to the predictive models of microfracture or osteotomy outcome were assessed to determine whether there was a differential concentration between individuals who were deemed ‘responders’ or ‘non-responders’ to either surgery.

Eight of the patients responded to microfracture surgery and 11 were non-responders based on a MCID of 10 Lysholm points. Figure 2a demonstrates that the SF concentration of none of the proteins (HA, YWHAQ, ADAMTS-4, LYVE-1) which contributed to the predictive model of microfracture outcome was significantly different between microfracture responders and non- responders. Despite the concentration of YWHAQ not being significantly different (p=0.18; Mann-Whitney) between responders (0±0 ng/ml)(mean(±SEM)) and non-responders (2.5±1.3 ng/ml (mean(±SEM)) to microfracture, it is interesting that in all the responders to microfracture surgery, YWHAQ was undetectable (n=8; Table 2) when assessed using ELISA but in clinical responders (n=11; Table 2) it was detected in only 50% of cases. Thus assessment of YWHAQ in the total patient cohort

|  |  |  |  |
| --- | --- | --- | --- |
| **Osteotomy (n=13)** | | | |
| **Component** | ***R2*  Value** | ***RMSE*** | **Variable Importance** |
| Total Model | .77 | 12.1 |  |
| Smoker |  |  | 100 |
| Age | 3 |
| ADAMTS-4 (ngml-1) | 2 |

**Table 7:** A generalized linear regression model with elastic net penalization for Predictors of the Post-Osteotomy Lysholm Score

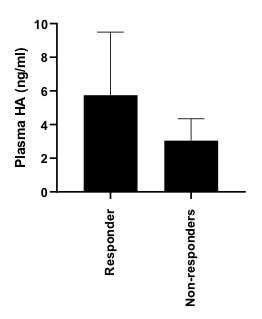
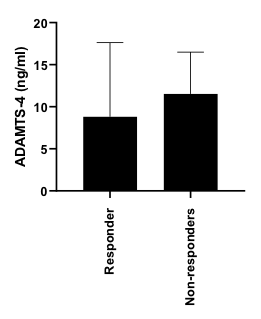
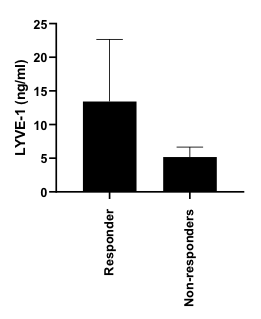
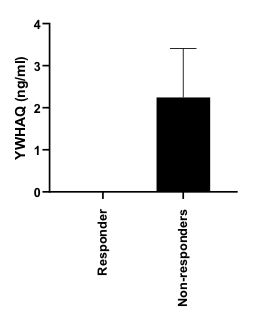
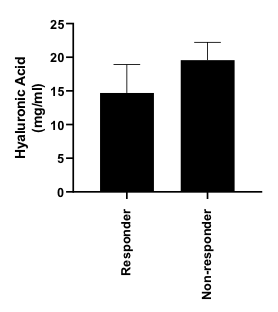
Final elastic net model parameters were alpha- 0.6, lambda- 5.2. Alpha is a value between 0 and 1, where 0 is pure ridge regression, 1 is pure LASSO and values between are a mixture of both. Lambda is the shrinkage factor applied to model coefficients

*Abbreviations: ADAMTS-4 (A disintegrin and metalloproteinase with thrombospondin motifs 4, RMSE (*Root mean square error)

confirms the findings of the proteomic analysis that YWHAQ is undetectable in responders to microfracture surgery.The concentration of HA within the plasma was not significantly different between the microfracture responders and non-responders (p>0.99).

Eight of the 13 patients treated with osteotomy improved by at least 10 points in the Lysholm score after one year and who were therefore classed as responders. The activity of ADAMTS-4 was not significantly different between individuals who responded and did not respond to osteotomy (Figure 2b) (responders: 0.4(±0) ng/ml, non-responders: 17.9(±7.3) ng/ml (mean(±SEM)); Mann-whitney; p<0.05). However all of the individuals who responded to osteotomy had undetectable activity of this enzyme within their SFs, whereas activity was detectable in three of the five non-responders.

**Figure 2:** Assessment of differential abundance/activity of candidate predictive markers in responders (n=8) cf. non-responders (n=11) to surgery. Concentrations of (A) hyaluronan (HA) (responders: 14.7±4.2 mg/ml, non-responders: 19.6±2.6 mg/ml (mean±SEM); p=0.52)), (B) 14-3-3 protein theta (YWHAQ) (responders: 0±0 ng/ml, non-reponders: 2.5±1.3 ng/ml (mean±SEM); p=0.18), (C) Lymphatic Vessel Endothelial Hyaluronan Receptor-1 (LYVE-1) (responders: 13.4±9.2 ng/ml, non-responders: 5.2±1.5 ng/ml (mean±SEM); p=0.918) in the synovial fluid (SF) were not different (Mann-whitney) between responders and non-responders to microfracture surgery. (D) Enzyme activity of A disintegrin and metalloproteinase with thrombospondin motifs 4 *(*ADAMTS-4) was not significantly different between responders (8.8±8.8 ng/ml (mean±SEM)) and non-responders (11.5±5.0 ng/ml (mean±SEM)) to microfracture surgery (Mann-whitney; p=0.76). Plasma HA concentration was not different between microfracture responders (5.7±3.8 ng/ml (mean±SEM)) and non-responders (3.0±1.3 ng/ml (mean±SEM)) (Mann-whitney; p>0.99).



**A**

**D**

**C**

**B**

**E**



**Figure 3:** None of the responders to osteotomy surgery demonstrated detectable enzyme activity of ADAMTS-4, however, enzyme activity was not significantly different between the responders (n=8) cf. non-responders (n=5) (responders: 0.4±0 ng/ml, non-responders: 17.9±7.3 ng/ml (mean±SEM); Mann-whitney; p=0.17). Data are mean±SEM.

**Discussion**

The need to identify predictive biomarkers that are able to recognise individuals who are most likely to receive clinical benefit from interventions aimed at delaying/preventing the development of OA has been highlighted as a research priority by the OARSI 16. This study aimed to identify novel candidate biomarkers with the potential to predict outcome to two of the most commonly used clinical procedures, microfracture and osteotomy. This is the first study, to our knowledge, to assess a panel of protein markers for their potential to predict patient outcome to these surgeries. We have previously identified candidate protein markers for the prediction of clinical outcome to the cell therapy, ACI 45. However, the work described here has the potential for more wide-spread application as microfracture and osteotomy surgeries are offered at a large number of hospitals, with more than 100,000 microfracture procedures in knees, hips or shoulders estimated to be carried out per year globally 7. These procedures provide cheaper alternative surgical options for the treatment of joint damage and early OA, which if used to treat the patients who are likely to benefit, can reduce the number of patients who will develop end-stage OA.

To identify novel candidate biomarkers we employed a two-pronged approach. The first was to utilise label-free quantitation proteomics in a non-targeted approach with the aim of identifying completely novel biomarkers that had not previously been associated with OA or cartilage damage/repair. The second, targeted approach was to assess a panel of SF and plasma proteins that we routinely test and that have either been previously associated with OA 31,45 or that we have identified as changed in response to ACI 11,12. This methodology meant that any candidate proteins identified from the proteomic analysis could be assessed in a larger cohort to determine whether they added any predictive value over and above other more commonly assessed ‘OA’ proteins, baseline OA severity (as deemed by Kellgren-Lawrence score) and patient demographics, together contributing to the development of predictive models for patient outcome to microfracture or osteotomy surgery. Further, particularly for the microfracture cohort there was a wide range of patient ages which in itself could account for differential abundance of some of the measured proteins e.g. serum HA19, therefore this statistical approach could account for co-linearity between parameters, such as between age and serum HA.

We acknowledge that one of the limitations of our study is that only a small number of samples were used in the proteomic analysis. This is because we wanted to select patients who demonstrated the worst and best clinical responses to either surgery, as determined by change in Lysholm scores between pre-operative and 12 month post-operative scores. Furthermore, the number of samples that could be included in these preliminary investigations was limited to those (i) that had sufficient volumes of SF, (ii) which were not too diluted (through the lavage procedure used to collect SF at our centre 31) so as to be loaded onto ProteoMinerTM  beads for dynamic range compression and (iii) that did not have any blood staining, as this has been demonstrated to alter the detection of SF proteins 44. Therefore, it is important that these results are not over interpreted. We did not undertake any bioinformatic pathway or network analyses on these proteomic findings due to the low number of samples and again to avoid over interpretation of the results. In addition, the markers identified in this preliminary proteomic study will need to be validated in larger independent osteotomy and microfracture cohorts.

One of the proteins identified in the proteomic analyses was 14-3-3 protein theta (YWHAQ). This protein was of particular interest for the prediction of microfracture outcome as it was undetectable (via LC-MS/MS or ELISA) in the SF of individuals who responded well to this procedure. The LC-MS/MS analysis also highlighted this protein as being detected only in the non-responders to osteotomy. However, two of the four samples used for proteomic analyses demonstrated detectable concentrations of YWHAQ when assessed using ELISA. This may be due to the sensitivity of the ELISA or perhaps that this proteins detection was masked due to other more abundant proteins in the proteomic analysis. We measured YWHAQ in the larger osteotomy patient cohort (n=13) from which this protein was not found to have predictive value between responders and non-responders to osteotomy. Therefore, we focussed upon the potential of this protein as a predictive marker for microfracture outcome. YWHAQ concentration was a significant variable in the linear regression model of microfracture outcome, indicating that with increasing pre-operative concentrations of YWHAQ, post-operative Lysholm score decreases. When divided into responders and non-responders to microfracture, based on a MCID of 10 Lysholm points, however, there was no statistical difference in concentration between the responder cohorts. It is notable that in the full cohort of microfracture patients (n=19) assessed, representing a more diverse range of clinical ‘response’ compared to those assessed in the proteomic analyses, none of the ‘responders’ SF samples had detectable levels of YWHAQ, whereas 55% of ‘non-responders’ had detectable concentrations. Therefore, there is value in testing this candidate biomarker protein in a larger cohort of individuals treated with microfracture. YWHAQ is a member of the 14-3-3 protein family which have a wide range of functions, largely related to signal transduction pathways 15. The role of this specific isoform is not clear, particularly in relation to its function in OA or cartilage tissue damage/repair. However, it has been identified in several other relevant proteomic studies which assessed (1) the membrane proteins of equine articular chondrocytes 23; (2) the secretome of cultured chondrocytes 29; (3) the SF of patients with either rheumatoid arthritis or OA 2 and (4) the SF of rabbits subjected to anterior cruciate ligament transection compared with sham injury 21. Together these data suggest that this protein may be secreted from chondrocytes into the SF but how changes in the abundance of this protein within the SF relate to OA disease severity/progression needs to be investigated further.

In this study we have started to develop clinical prediction models for microfracture and osteotomy by performing multiple regression analyses. Specifically, we generated a linear regression model that can correlate baseline SF HA, SF YWHAQ, SF ADAMTS-4, SF LYVE-1, and plasma HA concentrations along with patient smoker status and gender with Lysholm score at 12 months post treatment with microfracture. Moreover, although a weak correlation a generalized linear model with elastic net penalisation also generated for microfracture outcome which again highlighted SF HA concentration, SF ADAMTS-4 activity and baseline Lysholm score as predictors of post-microfracture Lysholm score. Furthermore, we generated a generalized linear regression model with elastic net penalisation which highlighted the activity of ADAMTS-4 enzyme in SF alongside patient smoker status and age as promising predictors of osteotomy outcome. These markers/prediction models need to be assessed in larger, independent cohorts to confirm their clinical utility.

Both regression models of microfracture outcome highlighted that lower pre-operative SF concentrations of HA are predictive of higher post-operative Lysholm score and hence better knee function/less pain following treatment with microfracture. HA is a non-sulfated glycosaminoglycan which forms an important component of articular cartilage and the synovial membrane and is highly concentrated in the SF 3. Altered concentrations of HA within the SF have long been considered a marker of degenerative joint disease 30 and serum HA levels can predict the progression of knee OA 36. Notably, baseline levels of HA were higher in individuals whose OA had progressed cf. those with earlier stage OA 36. Hence, we suggest that in our study, individuals who demonstrated better knee function following microfracture may have earlier stage OA (and lower concentrations of HA) contributing to their clinical success compared to others who had more progressive pre-operative OA changes (higher levels of HA) and poorer surgical outcomes. The potential utility of this marker for highlighting which individuals have more progressive OA pre-operatively is strengthened by the fact that despite pre-operative Kellgren-Lawrence score being included as a variable in the statistical modelling, it did not significantly contribute to the models prediction value. Moreover, there was not a significant difference in the baseline Kellgren-Lawrence scores between the microfracture responder and non-responder cohorts. Therefore, assessment of SF HA could perhaps be indicative of early OA severity level which is below the detectable level using radiography in this cohort. The immunoassay that we used in this study (Corgenix) is a sandwich protein binding assay that has microwells coated with a highly specific bovine hyaluronan binding protein to capture HA and uses an enzyme conjugated version of HA binding protein as a secondary antibody to detect and measure HA in the samples. Higher and lower molecular weight HA have different molecular properties and both have been assessed for therapeutic and prognostic use in OA 5,48. Therefore in future studies to investigate the potential of HA as a candidate predictive biomarker of microfracture outcome, assessment as to the contribution of each molecular weight variant of HA and the biological role this protein is playing would be of interest.

The regression models of osteotomy and microfracture outcome indicate that lower activity levels of ADAMTS-4 in the SF pre-operatively is a predictor of better patient-reported knee function following osteotomy or microfracture. This finding is akin to our previous work which demonstrated that the absence of detectable ADAMTS-4 in the SF is predictive of success following ACI 45. ADAMTS-4, also known as aggrecanase-1, is more active in the SF of individuals with OA 20,49. This enzyme cleaves large chondroitin sulphate hyaluronan-binding proteoglycans including aggrecan, a key structure of articular cartilage 33. Loss of aggrecan is a key driver in the progression of OA 4,34,39. Therefore increased/detectible activity of ADAMTS-4 in SF pre-operatively may indicate that these patients’ joints have OA which has progressed to a stage where realignment of the joint through treatment with osteotomy or stimulation of innate repair via microfracture surgery is insufficient to delay/halt the progression of their OA, explaining the propensity for their treatment to fail. Despite the model indicating that ADAMTS-4 activity is indicative of osteotomy outcome, when separated into responders and non-responders to this surgery based on a MCID of 10 Lysholm points there was no significant difference in ADAMTS-4 activity between the response cohorts. We suggest that when separated into osteotomy responder and non-responder categories, the difference in the activity of the ADAMTS-4 enzyme was not statistically significant due to the low numbers of patients within each arm, particularly as none of the patients that were classed as clinical responders had detectable enzyme activity, whereas activity levels were detectable in three of the five non-responders. The same situation could also have had an impact on the HA levels recorded. Since the initiation of this study, patients treated with osteotomy at our centre have not routinely completed Lysholm scores pre-operatively or at 12 months post-surgery, therefore deeming it difficult to increase the study numbers. We accept, however, that this is a limitation and therefore highlight the importance of independent validation.

In summary, we have generated a linear regression model that can be used to help predict Lysholm score following treatment with microfracture surgery. This model has highlighted a number of candidate SF and plasma biomarkers, alongside patient demographics which have the potential to predict microfracture outcome. . Further the activity levels of ADAMTS-4 in SF, alongside patient smoker status and age has the potential to predict the outcome following osteotomy. These protein markers and patient demographics have the prospective to categorise individuals into those likely to demonstrate functional improvement following these surgeries and hence the most appropriate surgical interventions can be offered to these patients, preventing the burden of treatment failure and the need to re-operate/provide an additional treatment. Alternatively, further research aimed at understanding the biological processes underlying the altered abundance of these proteins in individuals who respond poorly to these surgical interventions could provide novel therapeutic targets for the personalised augmentation of these surgeries, e.g. administering aggrecanase inhibitors coincidently alongside osteotomy surgery. These candidate predictive biomarkers need to be further validated in larger independent cohorts, however, this work provides a foundation for the identification of biomarkers to predict outcome to interventions aimed at delaying or halting the progression of OA.

***Declarations***

Ethics approval and consent to participate

SF and plasma were collected under two independent ethical approvals: ‘Investigating the potential for cells and molecules isolated from orthopaedic patients for modelling and understanding pathogenic conditions and developing diagnostic markers and therapies for musculoskeletal disorders and spinal cord injury’ (11/NW/0875); ‘and ‘Arthritis and cartilage repair study’ (06/Q6201/9). 11/NW/0875 was approved by the NRES committee North West- Liverpool East and 06/Q2601/9 was approved by Shropshire and Staffordshire-Shropshire local research ethics committee. All patients gave valid informed consent prior to their samples being collected.

Competing interests

The authors declare that they have no competing interests.

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