Purpose: Lubricin (aka. PG4, superficial zone protein) is secreted by synovial fibroblast and chondrocyte of superficial layer of articular cartilage, where it maintains a lubricated and friction-less surface. We speculated that that degradation lubricin by neutrophil elastase whose expression is up-regulated following inflammatory insults and the release of these fragments into the synovial cavity might be an early biomarker of joint diseases. We therefore set-out to develop an ELISA measuring protease derived fragments of lubricin.
Methods: Human cartilage and synovial membrane were in vitro digested by a variety of MMPs, aggrecanase, cysteine proteinase and serine proteinase respectively, including MMP2, MMP12, MMP13, ADAMTS4, ADAMTS5, Cathepsin B and Neutrophil Elastase. The proteolysis products were analyzed by mass spectrometry and evaluated using the Mascot database. A cleavage fragment (neoepitope) of lubricin was identified. A peptide was generated, which conjugated to KLH- and used as immunogen. Six eight-week old Balb/C mice were immunized sequentially and the immuno-reactivity towards the lubricin neoepitope was tested after 3rd, 4th and 5th immunization. Mice with best reactivity were selected and spleens were isolated and fused with SP2/O cells. The hybridomas were subsequently cloned by classic limiting dilution method, and subclones were produced. The best monoclonal antibody was selected based on following criteria: specificity towards neoepitope using a specific peptide (positive control), elongated peptide (negative control) and nonsense peptide (negative control). Furthermore, the Mabs were tested for reactivity in native samples, such as serum, urine, synovial fluid and culture supernatants. A competition ELISA was developed using the best Mab with following procedure: a 96-well streptavidin-coated plate was coated with 1ng/ml Biotin labeled lubricin neoepitope for 30 min at 20°C, at 300 rpm. The plate was then washed 5 times with standard wash solution. Standard and samples were added 20ul/well, followed by the addition of 100ul/well peroxidase labeled antibody and incubated onvernight (20±1 h) at 4°C, at 300 rpm. After that, the wells were washed 5 times and incubated with 100ul/well TMB at 20°C, at 300 rpm for 15 min. Then 100ul/well stop solution was added and the colorimetric reaction was measured at 450nm with reference at 650nm. The ELISA was used to characterize the release of the fragments from human cartilage explants.
Likewise, synovial fluid samples from OA patients were measured (n=15).
Results: A specific lubricin neoepitope with C-terminal cleavage site generated by Neutrophil Elastase was identified by mass spectrometry. Blasting results showed that it was specific for human lubricin. All mice had strong immunization response to the neoepitope linked KLH-immunogen. After serial subcloning, one antibody producing clone, Mab-13H1, with IgG [[Unsupported Character - K]] isotype was obtained and characterized. It had specific response to the specific peptide, but not toward the elongated peptide. Thus the Mab-13H1 was specific for the cleavage site. The competitive ELISA showed good technical performance: 1) measuring range from 0.27ng/ml to 200ng/ml; 2) intra- and inter-assay CVs: 10% and 15%; 3) Dilution recovery of human serum samples: 82.8%-112%; 4) interference: blocked by adding liquid-II. The neoepitope was found in synovial fluid of patients with range from 1,16ng/ml to 23,042ng/ml (unadjusted values).
Conclusion: We identified and developed a new competitive ELISA for measuring of lubricin neoepitope primarily from synovial membrane. We found measurable amounts in synovial fluid of RA patients, indicating that this could be a novel biomarker of early cartilage loss.

167

TWO NOVEL DIAGNOSTIC BIOMARKERS OF CARTILAGE DEGRADATION AND CONNECTIVE TISSUE INFLAMMATION ARE PREDICTIVE OF RADIOGRAPHIC DISEASE PROGRESSION

A.-C. Bay-Jensen [1], S. Wichuk [2], I. Byrjalsen [1], M.A. Karsdal [1], W.P. Maksymowych [2]. [1] *Nordic BioSci. A/S, Herlev, DENMARK;* [2] *Div. of Rheumatology, University of Alberta, AB, CANADA*

Purpose: Cartilage degradation and inflammation of connective tissue are key events in inflammatory and non-inflammaory arthropathies, such as osteoarthritis (OA), rheumatoid arthritis (RA) and ankylosing spondylitis (AS). Presently there are no prognostic tools available for measuring these tissue related processes, which are often the target of intervention. Inflammation induces an increase in collagenases. An elevated level of collagenases will lead to increased degradation of the disease affected tissues, e.g. cartilage and synovial tissue. Type II collagen is the primary protein component of cartilage and type III collagen is one of the major proteins of many connective tissues (e.g. synovial membrane); thus they are obvious targets for the action of collagenases. The objective of the study was to investigate whether those fragments could be used as predictive biomarkers of radiographic disease progression.
Method: Serum samples were collected from patient suffering from AS (n=124) at baseline and 2-3 month treatment follow-up. Standard AS clinical outcome scores were collected: BASDAI (health questionnaire) and mSASSS (radiographic scoring). Progressors were defined as having new vertebra syndesmophytes over a two year period. Serum levels of type II collagen degradation was measured by the C2M competitive ELISA, and type III collagen degradation by the C3M ELISA. Serum hsCRP was likewise measured. Logistic regression and CART were used to analyze the prognostic value of the markers individually or in combination.
Results: Both cartilage and connective tissue degradation fragments, C2M and C3M, were significantly elevated in AS patient serum samples compared to healthy controls ($p<0.0001$). The area under the curves of C2M and C3M, respectively, were 70% and 81% for AS. C3M correlated significantly with AS score BASDAI and mSASSS ($p<0.01$). C2M did not show the same correlations. A combination of the two markers could predict 80% of those who was defined as progressors, with a negative predictive value of 61%.
Conclusion: Present study is the first to show that the two novel biomarkers of cartilage and connective tissue degradation add additional information to the understanding of the diagnosis and progression of arthropathies. No other clinical score or biomarker possesses these properties.

Predictive value for radiographic progression of serum C2M and C3M

	High C2M (n=94)	High C3M (n=94)	High hsCRP (n=87)	High C2M & high C3M (n=33)
Positive predictive value	0.44 (0.27 - 0.62)	0.59 (0.41 - 0.75)	0.33 (0.17 - 0.53)	0.80 (0.52 - 0.96)
Negative predictive value	0.70 (0.57 - 0.81)	0.57 (0.43 - 0.69)	0.74 (0.60 - 0.84)	0.61 (0.36 - 0.83)

168

GLA RICH PROTEIN (GRP) IS ASSOCIATED TO OSTEOARTHRITIS BEING HIGHLY ACCUMULATED IN THE JOINT TISSUES AND SYNOVIAL FLUID

S.I. Cavaco [1], C.B. Viegas [1,2], R. Marta [1], R. Acácio [3], J. Silva [3], J.L. Morera [4], A. Teixeira [4], E. Smit [5], M. Herfs [5], C. Vermeer [5], D.C. Simes [1,2]. [1] *CCMAR, Algarve Univ., Faro, PORTUGAL;* [2] *GenoGla Diagnostics, Faro, PORTUGAL;* [3] *Faro Hosp., Dept. of Orthopedic and Traumathology, Faro, PORTUGAL;* [4] *Faro Hosp., Dept. of Anatomical Pathology, Faro, PORTUGAL;* [5] *VitaK, Maastricht Univ., Maastricht, NETHERLANDS*

Purpose: The newly discovered Gla-rich protein (GRP) is a vitamin K-dependent protein with high calcium-binding ability through its Gla residues. It was recently shown to be highly expressed and accumulated in rat cartilage and bone, and was suggested to be an important new player in the complexity of phenotypes involving connective tissue mineralization. With this work we aim to unveil the relationship between GRP and the molecular mechanisms involved in cartilage degradation and calcification associated with osteoarthritis (OA).
Methods: To investigate a possible involvement of GRP in OA processes we have analyzed samples of synovial membrane, cartilage, subchondral bone and synovial fluid, collected from OA patients at the time of total knee arthroplasty. Western blot and immunohistochemistry (IHC) techniques were performed using polyclonal antibodies against total GRP (CTerm-GRP) and the carboxylated form of GRP (anti-cGRP). Protein extracts from cartilage and synovial fluid were also analyzed using 2-D electrophoresis and GRP was detected by Western blot. The presence of the four GRP isoforms previously described in mouse was analyzed in human joint tissues using specific primers.

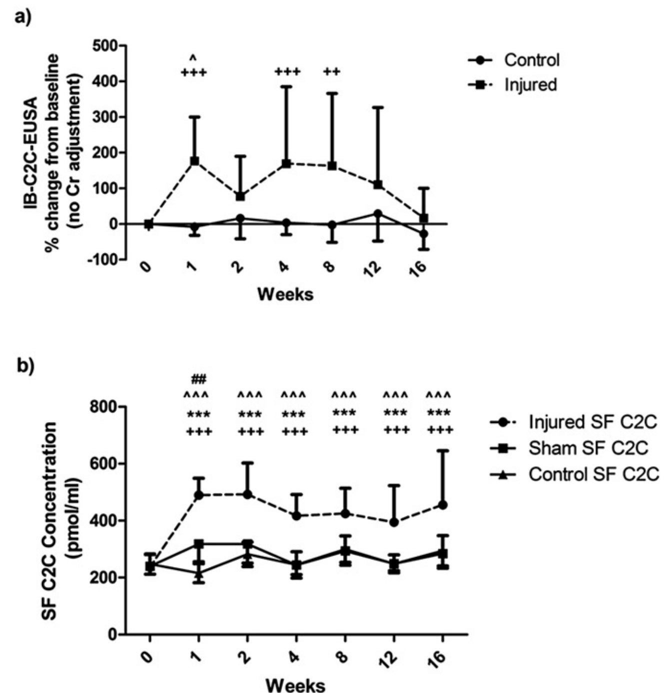
Results: GRP was detected by IHC throughout all mineralized areas in non-decalcified tissue samples, both in calcified cartilage and bone, as well as in the hyaline cartilage and chondrocytes. Decalcification of those tissue sections resulted in the loss of GRP signal associated with the mineralized matrixes, and clearly identified GRP within osteoblasts and osteocytes in bone, in agreement with GRP pattern of accumulation previously seen in rat skeletal tissues. Evidences for GRP accumulation in the osteoclasts were also seen, but this result needs further confirmation. In the synovial membrane, GRP was found highly associated with specific cell types at the synovial lining layer, within synoviocytes and in endothelial cells of capillaries and sporadically in some fibroblasts of the connective sublining layer. At sites of calcification GRP was detected co-localized with the mineral deposits. The detection of GRP in synoviocytes lead us to investigate the presence of GRP in the synovial fluid and the results obtained by Western blot analysis, either using the common or 2-D electrophoresis, pointed to the existence of several forms of GRP in this body fluid. Cartilage extracts analyzed by Western blot also showed the presence of several GRP forms. Altogether these results point to the existence of several forms of GRP in human. However, PCR amplifications using different sets of specific primers always resulted in the amplification of only the F1 isoform and no evidences were found for the existence of additional GRP spliced transcripts.
Conclusions: Our results show that in OA, GRP is accumulated in the articular and calcified cartilage, subchondral bone, synovial membrane and synovial fluid. The detection of GRP co-localized with the ectopic mineral deposits present in OA, confirms the association of this protein to pathological calcifications. Furthermore our results show that GRP is also highly accumulated at sites of inflammation associated with OA, and could indicate an active role of this Gla protein in inflammation processes. Although we were able to confirm the presence of isoform F1, we found no evidence for the existence in human of the other 3 alternatively spliced transcripts of the GRP gene (F2, F3 and F4) described to exist in mouse, suggesting that the different GRP forms detected by Western blot might correspond to different γ-carboxylation status of the protein.
At the moment a specific ELISA assay for GRP detection is being developed and will enable unveiling the relation between levels and forms of GRP in the synovial fluid with OA severity, providing a new tool that might be relevant in OA assessment and diagnosis.

169

NEW URINE BIOMARKER ASSAY COMPARES TO SYNOVIAL FLUID CHANGES AFTER ACUTE JOINT INJURY IN AN EQUINE MODEL OF OSTEOARTHRITIS

T.N. Trumble [1], D.M. Groschen [1], N. Ha [2], M. Boyce [1], K.A. Merritt [3], M.P. Brown [3]. [1] *Univ. of Minnesota, St. Paul, MN;* [2] *IBEX Pharmaceutical Inc, Montreal, QC, CANADA;* [3] *Univ. of Florida, Gainesville, FL*

Purpose: It has been well documented that collagenase cleavage of type II collagen occurs during osteoarthritis (OA) and that neoepitopes created by this cleavage can be identified and semi-quantified in the synovial fluid (SF) and serum. Recently, a new sandwich assay for urine (IB-C2C-EUSA; IBEX Pharmaceutical Inc) was developed that detects an intrachain epitope within 45 residues of the neoepitope on the Carboxy-terminal 3/4 fragment. The purpose of this study was to use this sandwich assay to determine if acute changes could be identified in equine urine following osteochondral (OC) injury and compare the findings to SF.
Methods: Twenty-two clinically and radiographically normal age- and sex-matched Quarter Horses were randomly divided into 2 groups: (1) horses (n=11) that had an OC fragment created arthroscopically in one randomly selected metacarpophalangeal (MCP) joint and a sham operation in the contralateral joint at week 0; and (2) unoperated exercise control horses (n=11). All horses were exercised on a treadmill from week 2 to week 16. Urine was collected via catheterization mid-morning in all horses. Blood was collected from the jugular vein and SF samples were collected without lavage from both MCP joints of all horses at baseline (week 0), and weeks 1, 2, 4, 8, 12, and 16. IB-C2C-EUSA kits were provided by IBEX. Other commercially available ELISA assays were purchased (C2C [IBEX], CTXII [IDS/Nordic], and MicroVue Creatinine [Quidel Corp]) and were used to analyze SF and serum or urine samples. IB-C2C-EUSA results were analyzed with and without creatinine (Cr) correction and the percent change from baseline was calculated. Normality was assessed and outliers removed. A repeated measures ANOVA with a Tukey's test for multiple comparisons was used for all analyses. Correlations between biomarkers were performed using Spearman Rank. $P \leq 0.05$ was considered significant.
Results: There was a significant effect of time (P=0.003), group (P=0.013) and the interaction between group and time (P=0.006) for the percent change from baseline of IB-C2C-EUSA concentrations that were not Cr adjusted (uC2C; Fig 1a). This is similar to SF C2C concentrations where there is also a significant effect of time, group, and the interaction ($P<0.0001$ for all; Fig 1b). With both assays, OC injured horses had greater change over time than the control group or shams, as demonstrated by IB-C2C-EUSA urine samples being significantly elevated above baseline and controls at week 1 and SF C2C samples being elevated above shams and controls from week1 to week 16 (Fig 1). Adjustment of IB-C2C-EUSA samples with Cr (as well as percent change) did not allow for any separation between groups. However, correlation analyses were similar regardless of whether Cr was adjusted for or not. Cr adjusted IB-C2C-EUSA concentrations were negatively correlated to age (R= -0.569; P=0.0001), SF C2C (R= -0.127; P=0.03), SF:serum C2C ratio (R= -0.152; P=0.009), and were positively correlated to urine CTX-II concentrations (R=0.685; P=0.0001).
Conclusions: Our results demonstrate that the IB-C2C-EUSA sandwich assay for equine urine can identify acute changes that occur in response to injury that is similar to the response identified in SF. In most equine biomarker studies, the only changes that occur within one week after injury to a single limb are in the SF. The results of this study suggest however, that IB-C2C-EUSA has the potential to identify these early changes as well, allowing for less invasive testing procedures. Further investigation will need to be performed to determine future recommendations regarding creatinine adjustment.
Figure 1. a) Percent change from baseline (±SD) for IB-C2C-EUSA concentrations (not Cr adjusted) for osteochondral (OC) injured and control horses. b) Synovial fluid C2C concentrations for OC injured and sham limbs, and controls. OC injured to baseline: +++ = $P<0.001$, ++ = $P<0.01$; OC injured to sham: *** = $P<0.001$; OC injured to controls: ^^^ = $P<0.001$, ^ = $P<0.05$; sham to control: ## = $P<0.01$.



170

SYNOVIAL FLUID BIOMARKERS OF INNATE IMMUNE ACTIVITY ARE PREDICTIVE OF KNEE OSTEOARTHRITIS PROGRESSION

P.A. Band [1], H.-G. Wisniewski [1], V. Liublinska [2], C. Pattanayak [2], E. Colon [1], J. Heeter [3], R. Karia [1], T. Stabler [4], E.A. Balazs [3], V. Kraus [4]. [1] *New York Univ.*