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Deliverable report on work package 4 (WP4), no. 3

Deliverable: Technical validation of pipeline BMs

Several new biomarkers (BM) were discovered and developed. It was decided that monoclonal antibodies were desired compared to polyclonal antibodies, which delayed the process for some of the assays. The table describes the BMs, which have been targeted and validated in the WP4. The usage of CIIM has been submitted as manuscripts to international peer review papers.

Ready-to-Use NB/SYNARC assays		
Assay	Short description	Assay specifications
CIIM-3.8 Type III collagen degradation and turnover Synovium marker	<ul style="list-style-type: none"> Measures protease-derived type III collagen fragments (competitive ELISA). Indications that circulating levels of the fragment are high in OA and RA patients Was previously called Helix-II, but a monoclonal antibody (vs. the original polyclonal) has been produced and a new protocol has been optimized In-house test shows elevated serum levels of the marker in RA patients (ref. Bay-Jensen) Kits have been produced for research use It is a 2nd generation biomarker (single neoepitope approach) 	<ul style="list-style-type: none"> Normal, healthy serum levels: <ul style="list-style-type: none"> Pre-menopausal women: 163.7 (94.1) ng/ml Post-menopausal women: 201.0 (168.4) ng/ml Men: 147.6 (6.9) ng/ml Intra- and inter-assay CV%: <11% Sample volume: 100 ul [1:10] for duplicates (10 ul raw sample) Lower limit of detection: 0.06 ng/ml Lower limit of quantification: 5.4 ng/ml Upper limit of quantification: 165 ng/ml
CIIM Type II collagen degradation by MMP Cartilage marker	<ul style="list-style-type: none"> Measures MMP-derived type II collagen fragments (competitive ELISA). Measures cartilage degradation A manuscript has been submitted on the characterization of this new biomarker (in urine). Another manuscript is being put together on the use of the assay in serum Kits have been produced for research use. It is a 2nd generation biomarker (single neoepitope approach) 	<ul style="list-style-type: none"> Normal, healthy urine levels: <ul style="list-style-type: none"> Pre-menopausal women: 83.02 (51.3) pg/mmol creatinine Post-menopausal women: 153.2 (156.4) pg/mmol creatinine Men: 84.8 (57.3) pg/mmol creatinine Intra- and inter-assay CV%: <6% Sample volume: 40 ul [1:2] for duplicates (20 ul raw sample) Lower limit of detection: 20 pg/ml Lower limit of quantification: 147 pg/ml Upper limit of quantification: 13400 pg/ml Both serum and urine samples can be used
342-G2 Aggrecan degradation	<ul style="list-style-type: none"> Measures MMP-derived aggrecan fragments (sandwich ELISA). Measures cartilage degradation Kits have been produced for research use 	<ul style="list-style-type: none"> Normal, healthy serum levels: <ul style="list-style-type: none"> Pre-menopausal women: 7.795 (2.750) ng/ml Post-menopausal women: 6.955 (1.934) ng/ml Men: 9.576 (2.200) ng/ml Intra- and inter-assay CV%: <10%

Cartilage marker	<ul style="list-style-type: none"> It is a 2nd generation biomarker (single neoepitope approach) 	<ul style="list-style-type: none"> Sample volume: 200 ul [1:10] for duplicates (20 ul raw sample) Lower limit of detection: 0.2 ng/ml Lower limit of quantification: 2.0 ng/ml Upper limit of quantification: 90 ng/ml
Upcoming NB/SYNARC Assays		
Assay	Short description	Assay specifications
373-G1 Aggrecan degradation by aggrecanase Cartilage marker (inflammation dependent???)	<ul style="list-style-type: none"> Measures aggrecanase-derived aggrecan fragments (sandwich ELISA). Measures cartilage degradation The assay is currently being characterized It is a 2nd generation biomarker (single neoepitope approach) 	NA (expected Q4-2010)
CIIC Type II collagen degradation by cathepsin Calcified Cartilage marker	<ul style="list-style-type: none"> Measures Cathepsin K-derived type II collagen fragments (competitive ELISA). Might be an important marker for bone involvement in cartilage degradation It is a 2nd generation biomarker (single neoepitope approach) 	NA (expected Q3-2010)
CIIM/β Type II collagen degradation by MMP combined with isomerization Aged cartilage degradation marker	<ul style="list-style-type: none"> Measures MMP9-derived fragments of aged type II collagen fragments (sandwich ELISA). Might be an important markers for OA, since it discriminate between old collagen and newly formed collagen, as well as taking into account the degradation observed in OA It is a 3rd generation biomarker (double neoepitope approach) 	NA (expected Q1-2011)

Publications:

1-5

1. Sumer, E.U. *et al.* MMP and non-MMP-mediated release of aggrecan and its fragments from articular cartilage: a comparative study of three different aggrecan and glycosaminoglycan assays. *Osteoarthritis Cartilage* **15**, 212-221 (2007).
2. Sumer, E.U., Qvist, P., & Tanko, L.B. Matrix Metalloproteinase and aggrecanase generated aggrecan fragments: implications for the diagnostics and therapeutics of destructive joint diseases. *Drug Dev Res* **68**, 1-13 (2007).
3. Bay-Jensen, A. *et al.* The MMP-derived type II collagen degradation marker, CIIM, is correlated to knee osteoarthritis – An ex vivo, serum, synovial fluid and immunohistochemical validation. *Semin. Arthritis Rheum* **In review**, (2010).
4. Charni, N., Richardor, P., Bay-Jensen, A., Delaisse, J.M., & Garnero, P. Nitrosylated N-telepeptide of type III collagen (IIIInys): a new specific biochemical marker of oxidative-induced synovial tissue metabolism in arthritis. *Osteoarthritis and Cartilage* **14**[B], S62. 2006.
Ref Type: Abstract
5. Wang, B. *et al.* Suppression of MMP activity in bovine cartilage explants cultures has little if any effect on the release of aggrecanase-derived aggrecan fragments. *BMC. Res. Notes*. **2**, 259 (2009).



A new biochemical marker of cartilage degradation: Measurement of MMP-derived type II collagen fragments in human urine

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Introduction:

Osteoarthritis (OA) is characterized by progressive damage of the joint tissue. During cartilage erosion, collagen type II and aggrecan, which are the most abundant molecules in the cartilage extracellular matrix (ECM), are sequentially degraded by proteolytical enzymes such as metalloproteinases (MMP). The resulting protein fragments, neopeptides, are released into the circulation and out to the urine, and could therefore potentially serve as biomarkers of cartilage destruction. We have developed an ELISA assay targeting the MMP generated type II collagen C-terminal neopeptide RDGAAG¹⁰⁵³, which we call CIIM. The assay is a competitive ELISA based on a monoclonal peroxidase labeled antibody (NB44-3C1).

Methods:

The 159 subjects were recruited at the Center for Clinical and Basic Research (CCBR) from the greater Copenhagen area, and had wide distributions of age, gender, BMI and varying degrees of OA symptoms at baseline. Subjects with inflammatory arthritis, any contraindication for MRI examination, or previous knee joint replacement were excluded from the study. Cartilage volume in the medial tibio-femoral compartment was quantified by a computer-based framework from magnetic resonance scans acquired using a Turbo 3D T1 sequence on a dedicated extremity Esaote scanner¹⁻⁹. 154 urine samples were thawed from -80°C storage and the level of CIIM and C-terminal telopeptide of type II collagen (CTX-II) was measured. Five samples were empty or missing from the biobank. The study was approved by the Danish National Committee on Biomedical Research Ethics (approval no. KA 2006-0054, Danish Ministry of Interior and Health). All participants signed approved information consent forms.

Results:

Technical performance of the CIIMB assay

Intra- and inter-assay CV% was 5.3 and 13.9, respectively and the quantification range was 147-14900 pg/ml with a lower limit of detection at 20 ng/ml. Reactivity towards native and MMP cleaved type II collagen was tested in the assay, and only cleaved type II collagen could displace the signal. Furthermore, cross reactivity was tested against peptides with similar to RDGAAG; only this specific sequence could displace the signal.

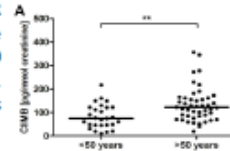
Measurement of urinary levels of CIIM in men and women (n=149)

Urinary CIIM levels, corrected to creatinine (CIIMB), were only weakly correlated with age and only for men. There was no correlation with body mass index (BMI) (table). The CIIMB level only correlated weakly with CTX-II in the male population (table).

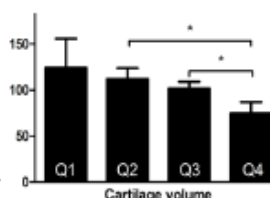
Gender	CIIMB (pg/mmol Creat.) Mean [95%-CI]	Age (years) Mean (range)	BMI (Kg/m ²) Mean (range)	CTX-II (pg/mmol Creat.) Mean (range)
Men (n = 82)	84.8 [72.2-97.0]	56.2 (22.7-76.9)	40.3 (22.0-86.8)	252.5 (40.5-1152)
		r = 0.265, p = 0.016 *	r = -0.069, p = 0.551	r = 0.218, p = 0.049 *
Women (n = 63)	126.4 [91.6-161.7]	56.3 (21.1-80.7)	25.7 (19.3-37.3)	240.2 (18.2-810.3)
		r = 0.209, p = 0.101	r = 0.037, p = 0.754	r = 0.090, p = 0.444
Diff. males/female	p = 0.0040			P = 0.3982

Urinary CIIM was higher in older than younger women

Urinary levels of CIIMB in the CCBR cohort. A) Comparison between the level of urinary CIIM in younger (<50 years, n= 27) and older (>50 years, n=48) women. The urinary levels were corrected to creatinine levels.



Urinary CIIM was correlated to Decreased cartilage volume



Cartilage volume measured by MRI and divided into quartiles. First quartile (Q1) contains urinary CIIM levels of the subjects with lowest cartilage volume and fourth quartile (Q4) subjects with the highest cartilage volume (n=134).

Conclusions:

Cartilage is a non-vascularized tissue consisting only of chondrocytes and ECM. The ECM is a composite network of proteins, primarily collagen type II. As the ECM is gradually broken down in joint degenerative diseases, such as OA, it presents a unique opportunity for targeting ECM specific biomarkers.

We have here presented data on a new biochemical and MMP-derived neopeptide marker of type II collagen - CIIM. This marker is correlated with cartilage volume and indirectly.

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