Investigation of two novel biochemical markers of inflammation, matrix metalloproteinase and cathepsin generated fragments of C-reactive protein, in patients with ankylosing spondylitis

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Abstract

Objectives

Ankylosing spondylitis (AS) is a chronic inflammation of the spine and the sacroiliac joints. Current markers of inflammation, such as C-reactive protein (CRP), are reflecting the production of an acute phase reactant rather than tissue specific inflammation, but the use of CRP as a diagnostic and prognostic marker for AS has not provided the sought accuracy and specificity. We hypothesized that local enzymatic activity in the disease-affected tissue, which is associated with extensive tissue turnover may, by cleavage, modify the CRP produced in the liver. These cleavage products may provide additional information on systemic inflammation as compared to that of full-length CRP. We investigated whether these CRP degradation products would provide additional diagnostic value in AS patients compared to full-length CRP.

Methods

CRP fragments were identified by mass-spectrometry. Two fragments were selected for ELISA development. One assay exclusively identified a matrix metalloproteinase (MMP) generated fragment, CRP-MMP, whereas the other assay identified a cathepsin generated fragment, CRP-CAT. Full-length CRP, CRP-MMP and CRP-CAT were measured in serum samples from 40 AS patients and 40 sex- and age-matched controls.

Results

Full-length CRP was not elevated in AS patients compared to controls, whereas CRP-MMP was elevated by 25% ($p<0.001$) and CRP-CAT by 50% ($p<0.0001$). The Area Under Curve of the Receiver-Operator Characteristic curve of CRP-CAT was the highest with 77%.

Conclusions

MMP and cathepsin degraded CRP provided more discriminative diagnostic potential compared to that of full-length CRP in this current study. These data suggest that different pools of CRP may provide insight into the inflammation processes in AS.

Key words

C-reactive protein, ankylosing spondylitis, biomarker, neoepitope, inflammation

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Introduction

C-reactive protein (CRP) is considered the prototypical acute phase reactant in human and is produced in response to a variety of clinical conditions including infection, inflammation and tissue injury (1, 2). During acute phase stimulus the serum concentration of CRP approaches a 1000 to 10.000-fold increase within 24-48 hours and decreases just as rapidly to the low normal concentration of a few μg/mL (1, 3). CRP is upregulated in both situations of acute and chronic inflammatory diseases, however it is a non-specific biochemical marker due to its upregulation in all inflammatory diseases (3).

CRP is synthesised by the liver and to some extend deposited in the inflamed tissue. The CRP sequestered in the inflamed tissue is susceptible to modification by the host cells, in the process of high tissue turnover (4, 5). The tissue modified versions of CRP may therefore provide a more tissue specific information compared to that of traditional full-length CRP. The target tissue may use several different mechanisms to modify proteins and CRP, including the protease battery produced during inflammation (4). Endopeptidases, such as matrix metalloproteinases (MMPs) and cathepsins, play a major role in the degradation of extracellular matrix proteins in many diseases (6), in particular spondylopathies and inflammation relation diseases (7-10) and have received increased attention for their proteolytic activity in generating disease specific fragments of proteins. These protein fragments, referred to as neoepitopes, are released into the circulation and have proven to be more accurate markers for certain pathophysiological processes during diseases (11). Specific proteolytic activities are a prerequisite for a range of cellular functions and interactions within the extracellular matrix (12). In pathological situations, including inflammation, the normal repair-response relationship is disturbed (13), leading to excessive protease secretion, tissue remodelling and release of a range of neoepitopes. These neoepitopes may be specific for the tissue of origin and for the involved proteases, and may consequently be used for design of molecular biochemical markers (11). For example, a type III collagen fragment generated by MMPs have been shown to be a marker for generalised and liver fibrosis (14, 15), type II collagen degradation by MMP has been demonstrated to be a marker for osteoarthritis and rheumatoid arthritis (16), and finally type I collagen fragments generated by cathepsin K, is a FDA approved diagnostic tool for measuring and monitoring bone resorption (11). In the specific context of CRP, fragments of CRP may provide additional information compared to that of full-length CRP.

Ankylosing spondylitis (AS) is a form of chronic inflammation of the spine and the sacroiliac joints (17). Destruction in AS starts at the entheses where several processes are observed: inflammation, bone destruction and new bone formation. One of the most commonly used monitoring parameters for AS in the serum is CRP, but this provides relative low sensitivity and specificity (18). Tissue remodelling with new bone formation is a central step in the pathogenesis of AS. This process has gained attention and several markers of the joint COMP, C2M, C2C (16, 19, 20) as well as regulators of bone formation, such as osteocalcin (21), DKK-1(22) and sclerostin (23) have been assessed in AS and have been linked to structural remodelling of the spine. These serological biochemical markers have provided preliminary insight into the disease processes of AS. However a link between inflammation and structural remodelling is so far lacking.

The current hypothesis is that protease-mediated degradation fragments of CRP occurs in AS, which may reflect the interplay between inflammation and tissue remodelling in AS. These neoepitopes could be more specific than full-length CRP, and allow gaining additional information for the diagnostic and prognostic discriminative power in patients with AS. This technology is referred to a protein fingerprinting, which have been documented for other pathologies to allow for better tissue accuracy compared to traditional full length proteins measurement by standard marker technologies (24-27).

The aim of the present work was to develop two ELISAs for quantitative assessment of protease degraded CRP by MMPs and cathepsin and to investigate whether these cleavage fragments provide additional information compared to full-length CRP in patients with AS.

Methods

In vitro cleavage of CRP

Purified CRP from human serum (Alpha Diagnostics) was cleaved with MMP-1, MMP-9, cathepsin K, cathepsin S (Calbiochem, VWR), MMP-3, MMP-8 (Abcam), A Disintegrin And Metalloproteinase with a Thrombospondin motif (ADAMTS)-1, and -8 (Abnova). The proteases were activated according to the manufacturers's instructions. Each cleavage was performed separately by mixing 200µg CRP and 2µg of activated enzymes in MMP buffer (100mM Tris-HCl, 100mM NaCl, 10mM $CaCl_2$, 2mM ZnOAc, pH 8.0), cathepsin buffer (50mM NaOAc, 20mM L-cystine, pH=5.5) or aggrecanase buffer (50mM tris-HCl, 10mM NaCl, 10mM $CaCl_2$, pH=7.5). As control 200µg CRP was mixed with MMP buffer only. Each aliquot was incubated for three days at 37°C. All MMP cleavages were terminated using GM6001 (Sigma-Aldrich) and all cathepsin and aggrecanase cleavages using E64 (Sigma-Aldrich). Finally the cleavage was verified by visualisation using the SilverXpress® Silver Staining Kit (Invitrogen) according to the manufacturers' instructions.

Peptide identification by MS

The cleavage products were purified and desalted using reversed phase (RP) micro-columns (Applied Biosystems) prior to nanoLC-MS-MS analysis as describes in litterature(28). The purified peptides were resuspended in 100% formic acid, diluted with H_2O and loaded directly onto a 18cm RP capillary column using a nano-Easy-LC system (Proxeon, Thermo Scientific). The peptides were eluted using a gradient from 100% phase A (0.1% formic acid) to 35% phase B (0.1% formic acid, 95% acetonitrile) over 43 min directly into an LTQ-Orbitrap XL mass spectrometer (Thermo Scientific). For each MS scan (Orbitrap, resolution of 60000, 300-1800Da range) the five most abundant precursor ions were selected for fragmentation (CID). The raw data files were converted to mgf files and searched in Mascot 2.2 using Proteome Discoverer (Thermo Scientific). Peptides with a mascot probability score $p<0.05$ were further analysed.

Selection of peptide for immunisations

The first six amino acids of each free end of the sequences identified by MS were regarded as a neoepitope generated by the protease in question. All protease-generated sequences were analysed for homology and distance to other cleavage sites and then blasted for homology using the NPS@: network protein sequence analysis (29).

Immunisation procedure

Six 4-6 week old Balb/C mice were immunised subcutaneously in the abdomen with 200µL emulsified antigen (50 µg per immunisation) using Freund's incomplete adjuvant (KAFVFPKESD-GGC-KLH and GNFEGSQSLV-GGC-OVA (Chinese Peptide Company, Beijing, China)). Immunisations were continued until stable titer levels were obtained. The mouse with the highest titer was selected for fusion and boosted intravenously with 50 µg immunogen in 100 µL 0.9% sodium chloride solution three days before isolation of the spleen for cell fusion. The fusion procedure has been previously described (30).

Characterisation of clones

The potential sequences KAFVFP and GNFEGS, named CRP-MMP and CRP-CAT respectively, were selected for antibody generation. Native reactivity and peptide binding of the monoclonal antibodies were evaluated by displacement of human serum in a preliminary indirect ELISA using biotinylated peptides (KAFVFPKESD-K-Biotin or GNFEGSQSLV-K-Biotin) on a streptavidin coated microtitre plate and the supernatant from the growing monoclonal hybridoma. Tested were the specificities of clones to the free peptide (KAFVFPKESD or GNFEGSQSLV), a non-sense peptide, and the elongated peptide (RKAFVFPKESD or GGNFEGSQSLV). Isotyping of the monoclonal antibodies was performed using the Clonotyping System-HRP kit (Southern Biotech). The selected clones were purified using Protein G columns according to manufacturer's instructions (GE Healthcare Life Science).

Assay protocol

The selected monoclonal antibodies were labelled with horseradish peroxidase (HRP) using the Lightning link HRP labelling kit according to the instructions of the manufacturer (Innovabioscience). A 96-well streptavidin plate was coated with 1.25ng/mL KAFVFPKESD-K-Biotin (CRP-MMP assay) or 0.40ng/mL GNFEGSQSLV-K-Biotin (CRP-CAT assay) dissolved in assay buffer (25mM Tris, 1% BSA, 0.1% Tween-20, pH 7.4) and incubated 30 minutes at 20°C. 20µL of free peptide calibrator or sample were added in duplicates to appropriate wells, followed by 100µL of conjugated monoclonal antibody (1A7-HRP or 3H8-HRP) and incubated 1 hour at 20°C. Finally, 100µL tetramethylbenzinidine (TMB) (Kem-En-Tec) was added and the plate was incubated 15 minutes at 20°C in the dark. All the above incubation steps included shaking at 300rpm. After each incubation step the plate was washed five times in washing buffer (20mM Tris, 50mM NaCl, pH 7.2). The TMB reaction was stopped by adding 100µL of stopping solution (1% HCl) and measured at 450 nm with 650 nm as the reference. A master calibrator, prepared from the synthetic free peptide accurately quantified by amino acid analysis, was used as a calibration curve and plotted using a 4-parametric mathematical fit model.

Technical evaluation and specificity

From 2-fold dilutions of quality control (QC) serum samples, linearity was calculated as a percentage of recovery of the 100% sample. The lower limit of detection was determined from 21 zero samples (*i.e.* buffer) and calculated as the mean + 3X standard deviation. The inter- and intra-assay variation was determined by 12 independent runs of 8 QC samples, with each run consisting

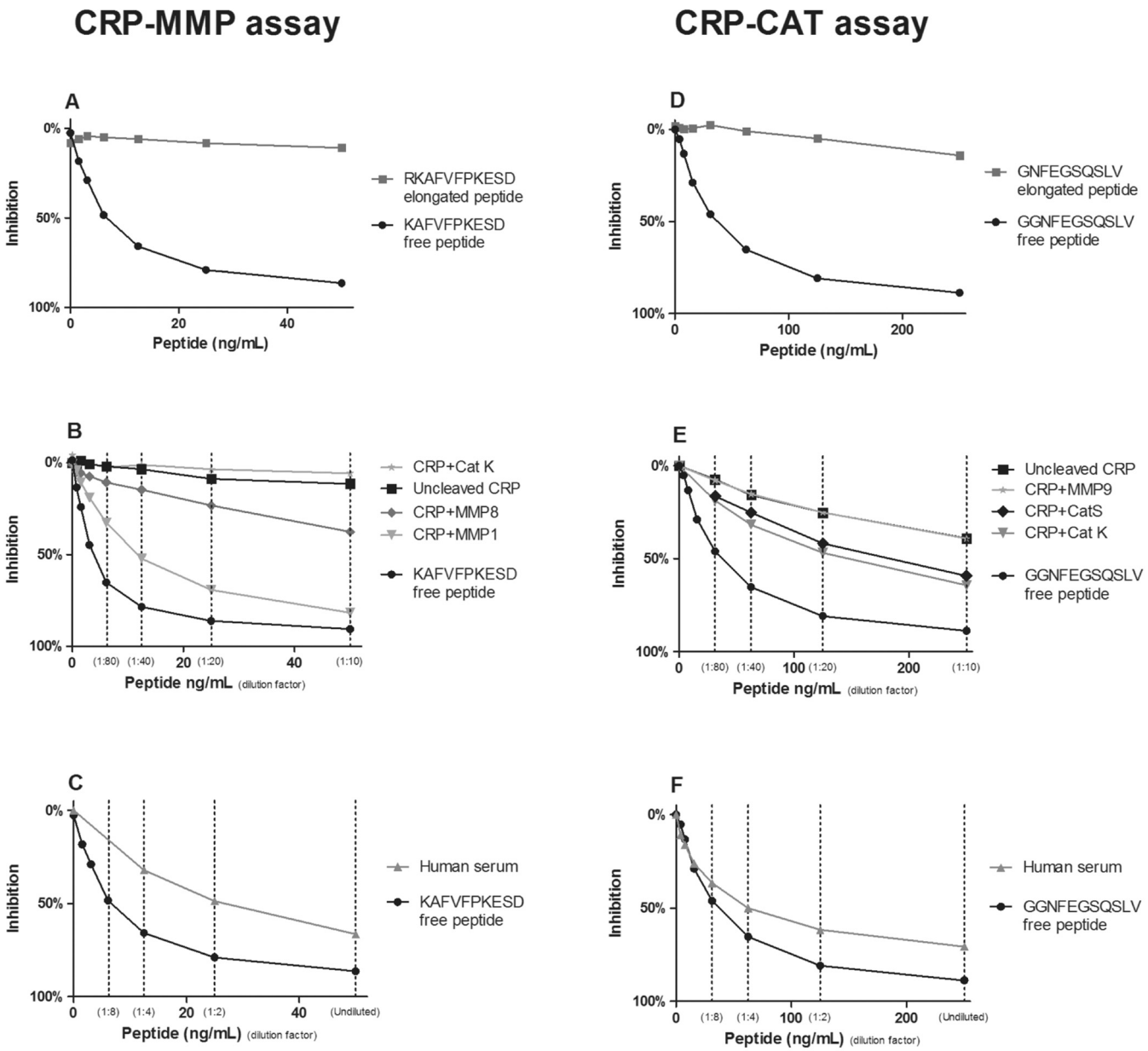

Fig. 1. Characterisation of the CRP-MMP (panel **A**, **B** and **C**) and CRP-CAT (panel **D**, **E** and **F**) monoclonal antibodies. ELISA showing percent inhibition of the signal of **A**) the free peptide and elongated peptide; **B**) the *in vitro* cleaved CRP with different MMPs and cathepsin K. The materials were run diluted 1:10, 1:20 and so on as indicated by the dotted lines; **C**) the free peptide and the native human serum sample which were run undiluted, diluted 1:2, 1:4 and so forth as indicated by the dotted lines; **D**) the free peptide and elongated; **E**) the *in vitro* cleaved CRP with different MMPs and cathepsin K. The materials were run diluted 1:10, 1:20 and so on as indicated by the dotted lines; **F**) the free peptide and the native human serum sample which were run undiluted, diluted 1:2, 1:4 and so forth as indicated by the dotted lines.

of two replicas of double determinations. The stability of serum samples was measured for three samples, which have been frozen and thawed for one to ten times.

The developed CRP-MMP and CRP-CAT ELISAs were evaluated using the materials described under "*In vitro* cleavage", where CRP was cleaved by different MMPs, cathepsins and aggrecanases. The materials were diluted 1:10 in the ELISA.

CRP-MMP, CRP-CAT vs. total CRP in patients with ankylosing spondylitis

CRP-MMP, CRP-CAT and full-length human CRP (Quantikine, R&D System) were assessed in serum from patients diagnosed with AS and compared to healthy sex- and age-matched controls from the Department of Medicine 3 of the University of Erlangen-Nuremberg. Serum samples were retrieved from patients diagnosed with ankylosing spondylitis (AS) according to the modified New York criteria and from sex- and age-matched non-diseased controls. BASDAI and mSASSS was registered for the each of the AS patients (Table II). The samples were diluted 1:4 in the CRP-MMP assay and in the CRP-CAT assay. The study was approved by the Ethics Committee of the University of Erlangen-Nuremberg and conformed to the principles outlined in the Declaration of Helsinki. Written informed consent was obtained from each person.

Table I. Summary table of the technical validation of CRP-MMP and CRP-CAT assays.

Technical validation step	CRP-MMP	CRP-CAT
Target	MMP degradation of CRP	Cathepsin degradation of CRP
Detection range	0.814-50.0ng/mL	7.28-250ng/mL
Dilution range of serum samples	1:2 - 1:4	1:2 - 1:4
Dilution recovery of human serum*	109%	106%
Intra-assay variation**	4.16%	5.01%
Inter-assay variation**	10.4%	6.77%
Analyte stability***	100%	98.4%

*Percentage dilution recovery was calculated as the mean of 5 human serum samples diluted 1:2, 1:4 and 1:8. **Inter- and intra-assay validation was calculated as the mean variation between 8 individual determinations of each human serum sample. ***The stability of the analyte (human serum) was calculated as the mean of three different serum samples, which were freeze and thaw for one to 10 times.

Table II. Demographic description of the study.

	Controls	AS
n. (women/men)	40 (21/19)	40 (19/21)
Age (range)	43.0 years (18 to 66)	42.5 years (29 to 63)
CRP [95% CI]	3016 ng/ml [1595, 4438]	4061 ng/ml [2426, 5696]
BASDAI		3.79 [0.01, 0.45]
mSASSS		5.08 [0.04, 1.14]

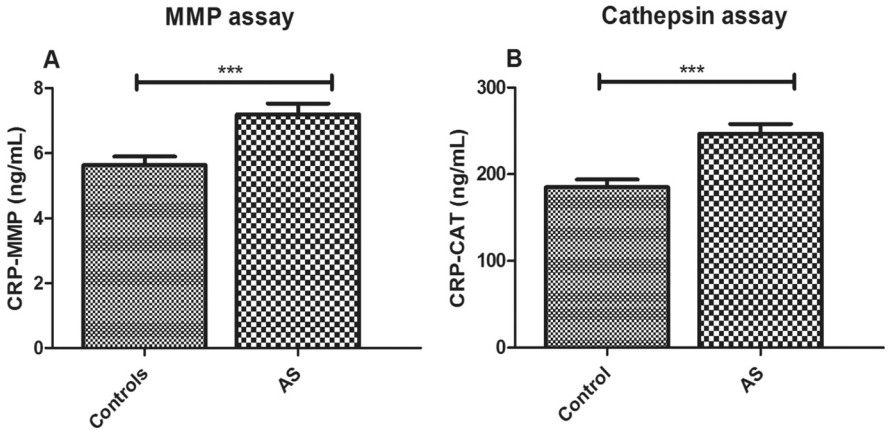

Fig. 2. Biological validation in human serum from patient with AS (n=40) compared with controls (n=40). **A**) CRP-MMP in serum in AS and controls, p=0.0007. **B**) CRP-CAT in serum in AS and controls, p<0.0001. Bars indicate mean level. Groups were compared by Wilcoxon rank sum test.

Statistics

Serum levels of the individual biomarkers between AS patients and non-diseased controls were compared using two-sided non-parametric Wilcoxon rank sum test. Correlations between the biomarkers were investigated by non-parametric Spearman's test. Area under the curve was measured with use of Receiver Operating Characteristic (ROC). The biomarkers were investigated in odds ratios (extrapolated from weighted levels: lowest value in the population was set at 0 and highest at 1) where all subject were classified as having normal (within SD of the mean of the normal population) or high (>SD) levels of the biomarker. Results were considered statistically significant if p<0.05.

Results

Assay development and validation

CRP was cleaved by a battery of proteases after which the fragments were identified by LC-MS/MS with a statistically significant MASCOT score. Four identified fragments were generated by MMP-1, 46 by MMP-3, 102 by MMP-8, 126 by MMP-9, 86 by MMP12, 38 by cathepsin K, 33 by cathepsin S, 7 by ADAMTS-1 and 8 by ADAMTS-8. All protease-generated neoepitopes were tested for homology and cross-reactivity to other proteins. Twenty of the most potential fragments were selected for immunisation and antibody generation

The requirements for selecting the monoclonal antibodies for ELISA development were I) IgG subtype, II) specific towards the neoepitope and not the elongated peptide nor uncleaved CRP, III) native reactivity towards human serum and not only the synthetic peptide and acceptable dilution recoveries of human serum. Based on these requirements two antibodies recognising the sequences KAFVFP and GNFEGS respectively were selected. The two monoclonal antibodies did not show any affinity toward either the elongated peptides or the uncleaved CRP (Fig. 1). The sequence KAFVFP was identified cleaved at the lysine 25 by MMP-1, -3, -8 and -9, and the sequence GNFEGS cleaved at the glycine 162 by cathepsin K, -S and ADAMTS-1. From the ELISA characterization it was observed that MMP-1 and MMP-8 were able to generate the KAFVFP fragment in an amount high enough to be detected by the ELISA. In contrast MMP-3, MMP-9 and cathepsin K were not able to generate the fragment (only cathepsin K shown in figure). Since the neoepitope is exclusively generated by MMPs, it is named CRP-MMP. Cathepsin S and K generated the GNFEGS fragment in an amount high enough to be detected by the ELISA. In contrast MMP-9 and aggrecanase-1 were not able to generate the fragment (only MMP-9 shown in figure). Since the neoepitope is exclusively generated by cathepsins, it is named CRP-CAT. The native reactivity towards human serum was high and the signal almost inhibited completely. These findings were consistent in the repeated cleaved batches.

The results of the technical evaluation are summarised in Table I, showing technically robust assays with dilution recovery within the recommended range of 10%. The accuracy and precision were acceptable with low inter- and intra-assay variation.

CRP degradation biomarkers are elevated in ankylosing spondylitis

Levels of the MMP generated neoepitope CRP-MMP were significant

(p=0.0007) higher in serum from patient with AS compared to controls (Fig. 2A). The levels of full-length human CRP in the AS samples compared to controls did not show any significant difference (data not shown). When measuring the cathepsin generated neoepitope CRP-CAT a significant difference (p<0.0001) was observed between the AS and controls (Fig. 2B).

Correlation between individual biomarkers

The correlations between the three measured biomarkers are shown in Table III. There was a significant correlation between CRP-MMP, CRP-CAT and full-length CRP in the AS group. The correlation between CRP-MMP and CRP-CAT was however stronger than between full-length CRP and the two degradation markers. The correlation in the controls was strong between CRP-MMP and CRP-CAT and low between full-length CRP and the two degradation markers. No correlation of the three individual markers to gender and age was observed (data not shown).

Diagnostic value of the biomarkers to differentiate between AS patients and controls

To investigate the diagnostic value of each biomarker, the ROC curves were made. The area under the curve (AUC) is calculated for the three biomarkers. CRP-CAT had the best diagnostic value (AUC 77%, p<0.0001) followed by CRP-MMP (AUC 72%, p=0.00066). The full-length CRP had the lowest diagnostic potential (AUC 63%, p=0.46). The odds ratios for differentiating controls from AS patients indicate that CRP-MMP is having the highest value (6.3, [2.0-20]) followed by CRP-CAT (3.9, [1.4-11]) (Fig. 3). Full-length CRP has the lowest value (1.7, [0.5-5.9]). The positive and negative predictive value is 0.48 and 0.88 for CRP-MMP, 0.45 and 0.83 for CRP-CAT and 0.20 and 0.88 for CRP.

Association to modified Stroke Ankylosing Spondylitis Spine Score (mSASSS) and Bath of Ankylosing Spondylitis Activity Index (BASDAI)

Up till now, there has been reasonable consensus about using BASDAI as the gold standard for disease activity in AS (18). No correlation between the three inflammatory biochemical markers and BASDAI was observed in this study. To investigate the relationship between the inflammatory biochemical markers and syndesmophyte formation in AS, the individual markers of CRP were correlated to the mSASSS score, which assesses the amount of syndesmophytes in the spin. Neither CRP-MMP, CRP-CAT nor full-length CRP correlated with the mSASSS.

Table III. The Spearman's correlations between individual biomarkers in AS group and controls.

	CRP	CRP-MMP	CRP-CAT
		Controls	
CRP		0.32*	0.32*
CRP-MMP	0.57***		0.81***
CRP-CAT	0.54***	0.75***	
		AS	

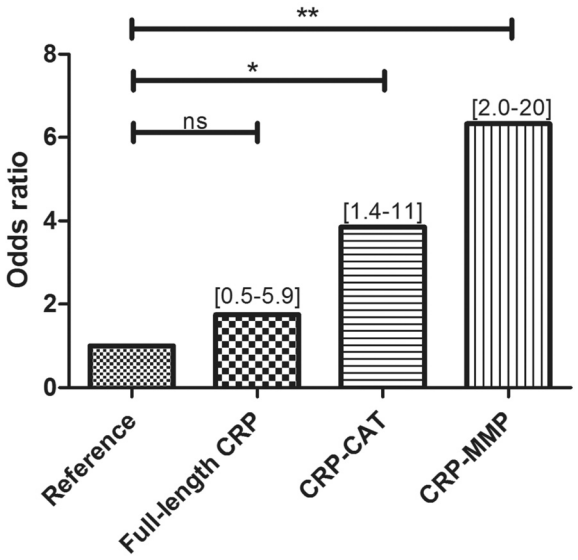

Fig. 3. Odds ratio for the three biomarkers in AS samples. Data is shown as mean±SD with 95% confidence intervals. The positive and negative predictive value is 0.20 and 0.88 for CRP, 0.48 and 0.88 for CRP-MMP and 0.45 and 0.83 for CRP-CAT.

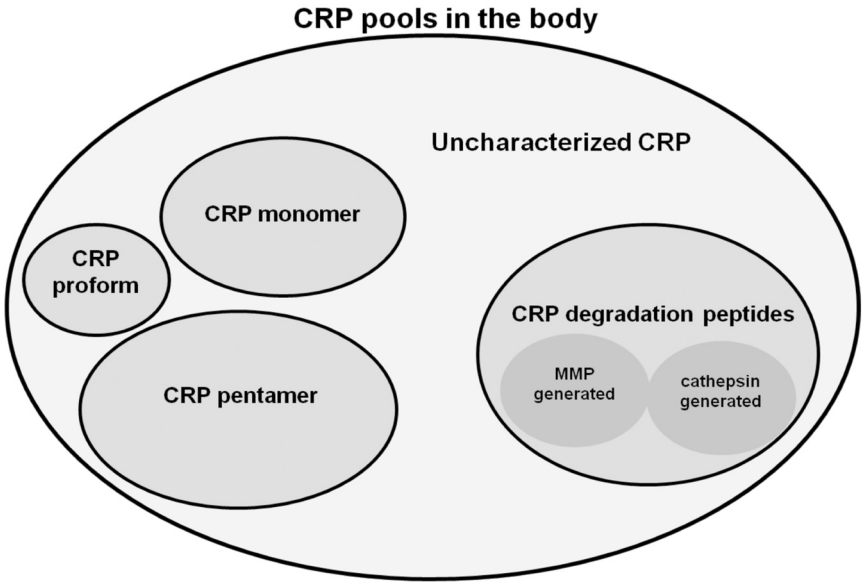

Fig. 4. The different CRP pools in the body.

Discussion

This is, to our knowledge, the first study to present assays detecting fragments of CRP generated by either MMPs or cathepsins. To identify the fragments of CRP, purified CRP from human serum was selected for cleavages *in vitro* by MMPs and cathepsins followed by analysis by MS. Such an approach has been undertaken for identification of other candidate biomarkers (6). The MS analysis identified a plethora of fragments and 20 specific fragments were selected for assay development that did not cross-react to other proteins and were predominantly generated by either MMPs or cathepsins. To be specific, it is important that the assay only recognise the peptide and not the elongated peptide containing an extra amino acid at the cleavage site nor the full-length CRP. Native reactivity is crucial, since it is human fluids and not synthetic peptide the assay is measuring. Out of the many developed assays, CRP-MMP and CRP-CAT assays meet these demands and have the potential of providing additional insight into the inflammation processes.

Endopeptidases such as MMPs and cathepsins play a major part in the degradation of extracellular macromolecules in diseases with inflammation and tissue destruction, such as AS (31, 32) and rheumatoid arthritis (RA) (33, 34). Indeed MMPs and cathepsins have been demonstrated to be the key players in a range of diseases with high extracellular matrix remodelling (5, 11, 16, 35, 36). In the current study highly significant differences between AS patients and age and sex-matched controls were observed when assessing both the CRP-MMP and CRP-CAT biomarkers. This significant difference was not observed in the full-length CRP nor others (18). CRP produced by the liver face a rapid degradation both in serum and in inflammation affected tissues, which could be the cause for the rather low CRP serum levels and lack of sensitivity in diseases like AS and other forms of spondylarthritides (SpA) as compared to RA (37). Moreover, the high local MMP and cathepsin activity could explain the elevated levels of CRP neoepitopes in patients with AS, although this need further investigation.

By further analysis we found that patients with a high CRP-MMP or a high CRP-CAT had increased odds ratios of 6.3 and 3.9, respectively, of having AS. Importantly, the AUC-values of both neoepitopes provide more diagnostic power in the current cohort compared to full-length CRP. Interestingly, the two cleavage products of CRP had higher correlation to each other than to full-length CRP in both controls and disease affected. The level of CRP-MMP and CRP-CAT were significantly lower than full-length CRP, even though they provided additional diagnostic information, suggesting that different sub-pools of CRP provides different types of information. Further analysis comparing the three types of CRP in additional diseases in which inflammation plays a major role may provide more specific details of the pathophysiological involvement of the different pools.

No correlation between the two CRP neoepitopes nor intact CRP with mSASSS and BASDAI was observed. Were mSASSS assesses the radiological damage of the cervical and lumbar spine that is associated with accumulated disease activity over time, inflammation markers are more the result of the current level of disease activity. In alignment, others studies also failed to demonstrate significant correlation of CRP to disease activity evaluated by *e.g* BASDAI and mSASSS (38, 39). This suggests that other types of markers may be more associated with the clinical score, such as structural markers of the joints (16) and syndesmophyte formation, such as osteocalcin (21). In future studies, particular emphasis should be directed to whether CRP-MMP and CRP-CAT could have prognostic potential for identification of fast progression that are in need of treatment, and predictive value of those that respond to a given treatment.

Biochemical markers measured in serum, plasma and urine are often products of local pathological events, in which many local specific events contribute to a particular pool of the marker. Even though some biochemical markers have been shown to correlate with the number of disease-affected sites, such as the correlation between cartilage degradation (40) and number of joint affected by the disease (41), tissue specificity and pathological relationships must be carefully evaluated. One approach for this evaluation may be by application of the Burden of disease, Investigatory, Prognostic, Efficacy and Diagnostic biomarkers (BIPED) criteria (11, 42) that was developed under the FDA critical path initiative by OsteoArthritis Research Society International (OARSI) members. Further studies are needed to investigate the clinical potential of these two CRP neoepitopes, and further classification according to the BIPED principle. Longitudinal studies are in particular warranted to investigate whether these two novel biochemical markers may provide any prognostic value, identifying fast progressors and the future course of the disease.

As illustrated in Figure 4, different forms of the same protein may be present in serum, in which each sub-pool holds unique information. This concept has been extensively applied to osteoporosis and bone turnover, in which the propeptide of type I collagen is used for bone formation (43) and the degradation fragment by cathepsin K (CTX-I) is use for bone resorption (36). For the current context this serves to highlight that different fragment of the same protein hold the completely opposite information. In alignment, aggrecanase and MMP mediated aggrecan degradation has been shown to reflect reversible and irreversible cartilage degradation, emphasising that two pools of the same protein entails unique biological information (13).

There is a range of limitations of the current study. Most importantly the small sample size and that the clinical assessment was done by mSASSS. mSASSS assesses the cervical and lumbar spine by radiography, thus it does not assess important AS associated events such as inflammation and joint health. These and other parameters may be better assessed by advanced MRI technologies. Furthermore, the preset study was a cross sectional study, which did not allow for assessment of the prognostic potential of this novel marker. The present finding

needs to be validated in a larger cohort. In conclusion, two robust assays have been developed using specific monoclonal antibodies for the detection of CRP-MMP, a CRP fragment derived from MMP activity and CRP-CAT, a CRP fragment derived from cathepsin activity in human serum. It was demonstrated that these two fragments were significantly elevated in AS patients while full-length CRP was not, illustrating that these CRP neoepitopes may provide additional diagnostic power than traditional biochemical markers. Further clinical studies are needed to evaluate the prognostic as well as the implication of these neoepitopes in other clinical inflammatory diseases.

References

1. VOLANAKIS JE: Human C-reactive protein: expression, structure, and function. *Mol Immunol* 2001; 38: 189-97.
2. DU CLOS TW: Function of C-reactive protein. *Ann Med* 2000; 32: 274-8.
3. HIRSCHFIELD GM, PEPYS MB: C-reactive protein and cardiovascular disease: new insights from an old molecule. *QJM* 2003; 96: 793-807.
4. CLUTTERBUCK AL, ASPLIN KE, HARRIS P, ALLAWAY D, MOBASHERI A: Targeting matrix metalloproteinases in inflammatory conditions. *Curr Drug Targets* 2009; 10: 1245-54.
5. SKJOT-ARKIL H, BARASCUK N, REGISTER T, KARSDAL MA: Macrophage-mediated proteolytic remodeling of the extracellular matrix in atherosclerosis results in neoepitopes: a potential new class of biochemical markers. *Assay Drug Dev Technol* 2010; 8: 542-52.
6. ZHEN EY, BRITTAIN IJ, LASKA DA *et al*.: Characterization of metalloprotease cleavage products of human articular cartilage. *Arthritis Rheum* 2008; 58: 2420-31.
7. YOSHIDA W, UZUKI M, NISHIDA J, SHIMAMURA T, SAWAI T: Examination of *in vivo* gelatinolytic activity in rheumatoid arthritis synovial tissue using newly developed *in situ* zymography and image analyzer. *Clin Exp Rheumatol* 2009; 27: 587-93.
8. HAMZAOUI K, MAITRE B, HAMZAOUI A: Elevated levels of MMP-9 and TIMP-1 in the cerebrospinal fluid of neuro-Behçet's disease. *Clin Exp Rheumatol* 2009; 27 (Suppl. 53): S52-S57.
9. KOSKINEN A, VUOLTEENAHO K, NIEMINEN R, MOILANEN T, MOILANEN E: Leptin enhances MMP-1, MMP-3 and MMP-13 production in human osteoarthritic cartilage and correlates with MMP-1 and MMP-3 in synovial fluid from OA patients. *Clin Exp Rheumatol* 2011; 29: 57-64.
10. MILNER JM, CAWSTON TE: Matrix metalloproteinase knockout studies and the potential use of matrix metalloproteinase inhibitors in the rheumatic diseases. *Curr Drug Targets Inflamm Allergy* 2005; 4: 363-75.
11. KARSDAL MA, HENRIKSEN K, LEEMING DJ *et al*.: Biochemical markers and the FDA Critical Path: how biomarkers may contribute to the understanding of pathophysiology and provide unique and necessary tools for drug development. *Biomarkers* 2009; 14: 181-202.
12. LOCHTER A, BISSELL MJ: An odyssey from breast to bone: multi-step control of mammary metastases and osteolysis by matrix metalloproteinases. *APMIS* 1999; 107: 128-36.
13. KARSDAL MA, MADSEN SH, CHRISTIANSEN C, HENRIKSEN K, FOSANG AJ, SONDERGAARD BC: Cartilage degradation is fully reversible in the presence of aggrecanase but not matrix metalloproteinase activity. *Arthritis Res Ther* 2008; 10: R63.
14. VEIDAL SS, VASSILIADIS E, BARASCUK N *et al*.: Matrix metalloproteinase-9-mediated type III collagen degradation as a novel serological biochemical marker for liver fibrogenesis. *Liver Int* 2010; 30: 1293-304.
15. BARASCUK N, VEIDAL SS, LARSEN L *et al*.: A novel assay for extracellular matrix remodeling associated with liver fibrosis: An enzyme-linked immunosorbent assay (ELISA) for a MMP-9 proteolytically revealed neoepitope of type III collagen. *Clin Biochem* 2010; 43: 899-904.
16. BAY-JENSEN AC, LIU Q, BYRJALSEN I *et al*.: 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: 423-9.
17. TAM LS, GU J, YU D: Pathogenesis of ankylosing spondylitis. *Nat Rev Rheumatol* 2010; 6: 399-405.
18. DE VLAM K: Soluble and tissue biomarkers in ankylosing spondylitis. *Best Pract Res Clin Rheumatol* 2010; 24: 671-82.
19. CONROZIER T, POOLE AR, FERRAND F *et al*.: Serum concentrations of type II collagen biomarkers (C2C, C1, 2C and CPII) suggest different pathophysiologies in patients with hip osteoarthritis. *Clin Exp Rheumatol* 2008; 26: 430-5.
20. KEMPTA LF, PIETTE JC, BASTUJI-GARIN S *et al*.: Serum cartilage oligomeric matrix protein (COMP) level is a marker of disease activity in relapsing polychondritis. *Clin Exp Rheumatol* 2010; 28: 553-5.
21. MUNTEAN L, ROJAS-VARGAS M, FONT P *et al*.: Relative value of the lumbar spine and hip bone mineral density and bone turnover markers in men with ankylosing spondylitis. *Clin Rheumatol* 2011; 30: 691-5.
22. PINZONE JJ, HALL BM, THUDI NK *et al*.: The role of Dickkopf-1 in bone development, homeostasis, and disease. *Blood* 2009; 113: 517-25.
23. APPEL H, RUIZ-HEILAND G, LISTING J *et al*.: Altered skeletal expression of sclerostin and its link to radiographic progression in ankylosing spondylitis. *Arthritis Rheum* 2009; 60: 3257-62.
24. GARNERO P, FERRERAS M, KARSDAL MA *et al*.: The type I collagen fragments ICTP and CTX reveal distinct enzymatic pathways of bone collagen degradation. *J Bone Miner Res* 2003; 18: 859-67.
25. KARSDAL MA, HENRIKSEN K, LEEMING DJ, WOODWORTH T, VASSILIADIS E, BAY-JENSEN AC: Novel combinations of Post-Translational Modification (PTM) neo-epitopes provide tissue-specific biochemical markers--are they the cause or the consequence of the disease? *Clin Biochem* 2010; 43: 793-804.
26. KARSDAL MA, WOODWORTH T, HENRIKSEN K *et al*.: Biochemical markers of ongoing joint damage in rheumatoid arthritis - current and future applications, limitations and opportunities. *Arthritis Res Ther* 2011; 13: 215.
27. KARSDAL MA, DELVIN E, CHRISTIANSEN C: Protein fingerprints - Relying on and understanding the information of serological protein measurements. *Clin Biochem* 2011; 44: 1278-9.
28. THINGHOLM TE, LARSEN MR: The use of titanium dioxide micro-columns to selectively isolate phosphopeptides from proteolytic digests. *Methods Mol Biol* 2009; 527: 57-66, xi.
29. COMBET C, BLANCHET C, GEOURJON C, DELEAGE G: NPS@: network protein sequence analysis. *Trends Biochem Sci* 2000; 25: 147-50.
30. GEFTER ML, MARGULIES DH, SCHARFF MD: A simple method for polyethylene glycol-promoted hybridization of mouse myeloma cells. *Somatic Cell Genet* 1977; 3: 231-6.
31. CHEN CH, LIN KC, YU DT *et al*.: Serum matrix metalloproteinases and tissue inhibitors of metalloproteinases in ankylosing spondylitis: MMP-3 is a reproducibly sensitive and specific biomarker of disease activity. *Rheumatology* (Oxford) 2006; 45: 414-20.
32. WENDLING D, CEDOZ JP, RACADOT E: Serum levels of MMP-3 and cathepsin K in patients with ankylosing spondylitis: effect of TNF-alpha antagonist therapy. *Joint Bone Spine* 2008; 75: 559-62.
33. SHIOZAWA S, TSUMIYAMA K, YOSHIDA K, HASHIRAMOTO A: Pathogenesis of joint destruction in rheumatoid arthritis. *Arch Immunol Ther Exp* (Warsz) 2011; 59: 89-95.
34. SCHURIGT U, STOPFEL N, HUCKEL M, PFIRSCHKE C, WIEDERANDERS B, BRAUER R: Local expression of matrix metalloproteinases, cathepsins, and their inhibitors during the development of murine antigen-induced arthritis. *Arthritis Res Ther* 2005; 7: R174-R188.
35. BARASCUK N, SKJOT-ARKIL H, REGISTER TC *et al*.: Human macrophage foam cells degrade atherosclerotic plaques through cathepsin K mediated processes. *BMC Cardiovasc Disord* 2010; 10: 19.
36. SORENSEN MG, HENRIKSEN K, SCHALLER S *et al*.: Characterization of osteoclasts derived from $CD14^+$ monocytes isolated from peripheral blood. *J Bone Miner Metab* 2007; 25: 36-45.
37. RUOF J, STUCKI G: Validity aspects of erythrocyte sedimentation rate and C-reactive protein in ankylosing spondylitis: a literature review. *J Rheumatol* 1999; 26: 966-70.
38. SHEEHAN NJ, SLAVIN BM, DONOVAN MP, MOUNT JN, MATHEWS JA: Lack of correlation between clinical disease activity and erythrocyte sedimentation rate, acute phase proteins or protease inhibitors in ankylosing spondylitis. *Br J Rheumatol* 1986; 25: 171-4.
39. SPOORENBERG A, VAN DER HD, DE KE *et al*.: Relative value of erythrocyte sedimentation

rate and C-reactive protein in assessment of disease activity in ankylosing spondylitis. *J Rheumatol* 1999; 26: 980-4.

40. SONDERGAARD BC, HENRIKSEN K, WULF H *et al*.: Relative contribution of matrix metalloprotease and cysteine protease activities to cytokine-stimulated articular cartilage degradation. *Osteoarthritis Cartilage* 2006; 14: 738-48.
41. MEULENBELT I, KLOPPENBURG M, KROON HM *et al*.: Urinary CTX-II levels are associated with radiographic subtypes of osteoarthritis in hip, knee, hand, and facet joints in subject with familial osteoarthritis at multiple sites: the GARP study. *Ann Rheum Dis* 2006; 65: 360-5.
42. BAUER DC, HUNTER DJ, ABRAMSON SB *et al*.: Classification of osteoarthritis biomarkers: a proposed approach. *Osteoarthritis Cartilage* 2006; 14: 723-7.
43. LEEMING DJ, LARSEN DV, ZHANG C *et al*.: Enzyme-linked immunosorbent serum assays (ELISAs) for rat and human N-terminal pro-peptide of collagen type I (PINP)--assessment of corresponding epitopes. *Clin Biochem* 2010; 43: 1249-56.