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Effect of tocilizumab combined with methotrexate on circulating biomarkers of synovium, cartilage, and bone in the LITHE study

Anne C. Bay-Jensen, MSc, PhD[a,*], Adam Platt, PhD[b], Inger Byrjalsen, MSc, DrSci[a], Philippe Vergnoud, MSc, PhD[c], Claus Christiansen, MD, PhD, DrMed[a], Morten A. Karsdal, MSc, PhD[a]

[a] Nordic Bioscience, Herlev Hovedgade, DK-2730 Herlev, Denmark
[b] Roche Products Ltd, Welwyn Garden City, United Kingdom
[c] Synarc Laboratories, Lyon, France

A B S T R A C T

Objective: We investigated the effects of tocilizumab (TCZ) on joint tissue remodeling in patients with moderate to severely active RA by measuring tissue-specific biomarker.
Methods: The LITHE biomarker study ($n = 740$) was a phase III study of 4- and 8-mg/kg TCZ in combination with MTX. Early response was evaluated at week 16 as $\pm 20\%$ improvement in swollen/tender joint counts; and ACR50 was evaluated at week 52. Biomarkers (tissue inflammation: C3M, CRPM, and VICM; cartilage degradation: C2M; and bone turnover: CTx and osteocalcin) were tested in serum from baseline, week 4, 16, 24, and 52, and dose-dependent effect was investigated. Patients were divided into the following three groups: early non-responders (ENR), ACR50 responders, and non-responders; their biomarker profiles were compared.
Results: At week 52, CRP was inhibited to 4% and 40% of baseline by TCZ8 and TCZ4, respectively. CRPM (63%), C2M (84%), C3M (69%), and VICM (42%) were significantly ($p < 0.05$) reduced by TCZ8, but not by TCZ4. MMP3 and osteocalcin changed to $< 58\%$ and $> 111\%$, respectively, in response to TCZ. CTx was not changed significantly. ENRs had significantly less inhibition of CRPM ($p < 0.05$), C2M ($p < 0.01$), and C3M ($p < 0.01$) compared to early responders. There was a significant difference in the C2M, C3M, and CRPM profiles of the ENRs, non-responders, and responders. ACR50 responders had significantly inhibited levels ($p < 0.001$), irrespective of dose.
Conclusions: TCZ8 strongly inhibited the biomarkers of joint tissue remodeling suggesting that TCZ actively suppresses key pathobiological processes at the site of inflammation in RA patients. The differences in biomarkers' profiles of responders and non-responders indicate that specific responder profiles exist.

Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune disease that is characterized by poly-articular inflammation associated with synovitis, osteitis, and periarticular osteopenia, often associated with loss of cartilage and erosion of subchondral bone. These features commonly lead to progressive joint damage, impaired function, and disability [1,2]. The challenges in the RA field have shifted from finding efficacious treatment options to identification of those patients who will benefit the most from treatment. It may therefore be critical to identify biomarkers or a panel of biomarkers that are diagnostic and predictive.

In RA, it is the persistent burden of pro-inflammatory cytokines, such as interleukins (e.g., IL-1, IL-6, and IL-17) and tumor necrosis factor alpha (TNFα), secreted by monocytes, T, and B cells, which drives disease progression. This leads to activation of the several signaling cascades, such as janus kinases (JAK), spleen tyrosin kinase (SYK), and mitogen-activated protein (MAP) kinases, ultimately resulting in the secretion of proteolytical enzymes, such as matrix metalloproteinases (MMPs), which are the main mediators of tissue

Abbreviations: ACR, American College of Rheumatology; ANOVA, analysis of variance; AUC, area under the concentration–time curve; BMI, body mass index; CRP, C-reactive protein; CTx, C-terminal cross-linking telopeptide of type I collagen; CV, coefficient of variation; DAS, disease activity score; DMARD, disease-modifying anti-rheumatic drug; ELISA, enzyme-linked immunosorbent assay; ESR, erythrocyte sedimentation rate; EULAR, European League against Rheumatism; JSN, joint space narrowing; IL-6, interleukin-6; MTX, methotrexate; OC, osteocalcin; RA, rheumatoid arthritis; SD, standard deviation; SEM, standard error of the mean; sIL-6R, soluble IL-6 receptor; TCZ, tocilizumab.

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* Corresponding author.
E-mail address: acbj@nordicbioscience.com (A.C. Bay-Jensen).

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destruction [3]. While the variation in the individual cytokines involved in the process and consequent signaling pathways may be large, the common end product is tissue destruction by cleavage of joint-specific proteins mediated by the increase in the proteases [4]. Thus measurement of end products of tissue destruction may provide more value than measurement of individual proteases and cytokines.

The elevated proteolytic activity results in the release of protein-specific tissue fragments and neo-epitopes, which have been investigated as biomarkers of joint disease [5]. Validated examples of neo-epitope biomarkers are those released following cleavage of type II collagen by MMPs, which reflect cartilage degradation. Among these biomarkers are serum MMP-degraded type II collagen (C2M) [6], urinary C-terminal telopeptide (CTX-II) [7–9], and the one-quarter fragment (C2C, TIINE) [10]. Other connective tissues, such as the synovium, are modulated by proteases, and neo-epitope biomarkers of synovium have also been identified. One such serum marker is C3M, which measures specific fragments of type III collagen and has recently been associated with inflammation-driven tissue turnover and fibrosis [11,12]. Measurement of serum CRP, an acute-phase reactant produced in the liver, is considered an excellent tool for diagnosing acute inflammatory diseases, but has little prognostic or predictive value [13]. Recently, a newly developed CRP measure was described. CRPM (i.e., CRP degraded by MMP) can be measured in serum to quantify CRP fragments released from the inflamed tissue, after CRP has been synthesized in the liver and deposited in the joint and degraded in the joint by the proteolytic burden [14].

Interleukin-6 (IL-6) is a pleiotropic cytokine involved in several pathways of inflammation and in bone and cartilage metabolism in RA [15]. Tocilizumab (TCZ) is a humanized anti-IL-6 receptor (IL-6R) monoclonal antibody. The effects of TCZ on bone turnover markers have been examined in several other RA clinical studies, including RADIATE [16] and OPTION [17]. These studies showed that both 4- and 8-mg/kg TCZ inhibited bone resorption significantly, whereas bone formation, measured by serum osteocalcin, was induced only by 8 mg/kg. This indicates that there are marked differences between the two approved doses (in US and EU, respectively). Analysis of structural changes by radiography showed that the two doses were equally protective against joint destruction [18], which highlights the need for further investigation of differences between doses.

This report summarizes the efficacy of exploratory biomarkers measured in the pivotal phase III called LITHE [18,19]. The LITHE study was designed to evaluate the effects of TCZ, 4 mg/kg + methotrexate (MTX) or 8 mg/kg + MTX, and placebo + MTX in RA patients with moderate to severe disease and non-responsive to DMARD. The aim of the current serological biomarker analysis was to investigate the effect of the two approved doses of TCZ, 4 mg/kg and 8 mg/kg compared to placebo, on the release of specific and descriptive biomarkers reflecting joint deterioration and inflammation. The biomarkers were C2M (a measure of cartilage degradation), C3M (indicating synovial inflammation), MMP3, total CRP, CRPM (tissue inflammation), citrullinated and MMP-degraded vimentin (VICM) [20], osteocalcin (assessing bone formation), and C-terminal telopeptide of type I collagen (CTX, indicating bone resorption). A further aim was to investigate each biomarker's predictive value for early and late clinical response to the intervention and thus potentially identify unique responder and non-responder profiles.

Patients and methods

Study design and serum samples

The LITHE have previously been thoroughly described by Kremer et al. [19] and Smolen et al. [18] (clinicalTrials.gov identifier: NCT00106535). The study is a 2-year phase III, multicenter, randomized, 3-arm, placebo-controlled, and parallel group trial in patients with moderate to severely active RA who had an inadequate response to MTX. The sub-study of LITHE consisted of serum samples from a 1-year, double-blinded treatment study, where 704 patients were randomized 1:1:1 to one of three treatment groups: 4-mg/kg, 8-mg/kg TCZ, or placebo (PBO) in combination with a stable dosage of MTX (10–25 mg/week). TCZ and PBO were given intravenously over 4 weeks. The end point of the biomarker sub-study was response rate, according to the American college of rheumatology criteria, for 50% improvement (ACR50).

Patients who failed to respond to treatment during the study, that is, they experienced $<20\%$ improvement from baseline in the swollen joint counts (SJC) and tender joint counts (TJC) at week 16 or later, they could receive blinded rescue therapy in a stepwise fashion between weeks 16 and 28, as follows: in the first-step blinded rescue, patients receiving PBO + MTX switched to TCZ 4 mg/kg + MTX, patients receiving TCZ 4 mg/kg + MTX switched to TCZ 8 mg/kg + MTX, and patients receiving TCZ 8 mg/kg + MTX remained on TCZ 8 mg/kg + MTX. Second-step rescue consisted of TCZ 8 mg/kg + MTX for all patients, regardless of initial treatment, and was offered through week 52, if inadequate response persisted after three doses of first-step rescue therapy. Patients who did not respond after three doses of second-step rescue discontinued treatment. Patients receiving rescue treatment were designated as early non-responders (ERN) for the purpose of the primary biomarker analyses.

In the study protocol, serum for biomarker research was scheduled to be collected from patients who provided written informed consent. Thus, the LITHE biomarker study was a prospective study. Because the primary objective of the current exploratory study was to examine the effect of IL-6R inhibition with TCZ on biochemical marker levels, only patients who provided a baseline sample, before initiating therapy and at least one post-dosing sample at 2, 4, 16, 24, and 52 weeks were included. The blood was collected in the morning after an overnight fast of >8 h at baseline, weeks 4, 16, 24, and 52, while non-fasting blood was collected at week 2. All samples were stored frozen at a temperature below -70°C until assayed.

The study was approved by the ethics committee at each participating institution and was conducted according to the Principles of Good Clinical Practice and according to the Declaration of Helsinki. All patients provided written informed consent. No steering committee was used for this study.

Biochemical marker assays

Serum levels of C2M [12], C3M [11], CRPM [14], and VICM [20] were measured blindly by manual competitive ELISAs, developed by Nordic Bioscience (Herlev, Denmark). Briefly, for C2M, which measures MMP-degraded type II collagen fragments; 4 ng/mL of biotin-KPPGRDGAAG (American peptide, Sunnyvale, CA, USA) was coated onto the streptavidin pre-coated 96-well plate (Roche Diagnostics, Mannheim, Germany) and left for 30 min at 20°C. After washing (PBS + 10% tween 20), the calibrators, controls, and undiluted serum samples were added followed by peroxidase-conjugated monoclonal antibody NB44-3C1, and incubated at 4°C overnight. The sample–antibody mix was washed off the well plate and peroxidase reaction was visualized by 3,3′,5,5′-tetramethylbenzidine (TMB, Kem-En-tec, Taestrup, Denmark) at 20°C and stopped with sulfuric acid after 15 min. For C3M, which measures the MMP-derived type III collagen neo-epitope, 96-well streptavidin-coated plates were coated with 0.4 ng/mL of KNGETGPQGP-biotin and left for 30 min at 20°C. After washing, calibrators, controls, and serum samples (diluted 1:1 in incubation buffer)

were added, followed by peroxidase-conjugated antibody NB51-G12. The sample–antibody mix was incubated at 20°C for 60 min. TMB was added after washing off the plates and incubated at 20°C and stopped with sulfuric acid after 15 min. CRPM measurement followed the same procedure as C3M; however, applying a different peroxidase-conjugated antibody (NB94-1A7) and coater (KAFVFPKESDK-biotin). For measurement of serum, VICM samples were prediluted 4 times in incubation buffer. Streptavidin-coated 96-well plates were coated with 100 μL of 1-ng/mL biotin–RLRSSVPGV–Citrulline and left for 30 min at 20°C. After washing, the calibrators, controls, and prediluted serum samples were added followed by 100 μL of peroxidase-conjugated monoclonal antibody NB212-1C5 and incubated at 4°C overnight. After sample/calibrator incubation, the wells were washed and incubated with 100 μL of TMB at 20°C for 15 min, followed by the addition of 100-μL stop solution (sulfuric acid) in each well. The colorimetric reaction was measured at 450 nm with reference at 650 nm using the Softmax Pro®, version 5 software (Molecular Devices, Sunnyvale, CA, USA). Intra- and inter-assay variations (CV) were below 8% and 10%, respectively, for all the above described assays.

Serum total MMP3 was measured by a two-site ELISA using two polyclonal antibodies raised against human MMP3 (Quantikine®, R&D systems, Denmark). Intra- and inter-assay CVs were $< 10\%$. Serum osteocalcin (OC) and CTX were measured by an automated multiplex assay (IMPACT bone chip, Roche Diagnostics, Mannheim, Germany) using the same antibodies as those employed in the corresponding single-marker assays (Elecsys, Roche Diagnostics, Mannheim, Germany) [21].

Statistical analyses

Summary statistics of general demographics, baseline RA characteristics, and baseline ACR demographics (Table 1) included the number of patients and the mean and standard deviation. The primary analysis (Fig. 1) was based on the mean percentage ($\pm$SEM) relative to the baseline measurement of the biochemical markers separated into the treatment. Difference between the two treatment groups, TCZ 4 mg/kg (TCZ4 + MTX) and TCZ 8 mg/kg (TCZ8 + MTX), and placebo (placebo + MTX) group was assessed by one-way ANOVA at weeks 16 and 52 on log transformed data. The *p*-values were adjusted for multiple comparisons by the Dunnett test.

For markers showing significant and consistent associations in the primary analysis, further associations between change in biomarker at week 4 and response at week 16 were investigated using a dichotomous approach in which patients were categorized according to designation of "high" and "low" levels, and "responders" and "non-responders." Sensitivity and specificity, including AUCs, were used to determine the optimal cut-off values derived from receiver–operator curve (ROC) analyses at highest likelihood ratio. Odds ratios were calculated using these cut-off values. Difference between responder and non-responder groups (Fig. 2) was investigated by Student's *t*-test.

All statistical analyses were performed using SAS software version 9.1.3 and MedCalc version 12.3.0. Graphing was performed using Prism Graphpad version 5.03.

Results

Characteristics of the patient participating the LITHE sub-study

The full trial description is given by Kremer et al. [19]. The LITHE study had approximately 400 patients in each treatment arm, of which 82–84% were females with an average age of 52 years and mean disease duration of 9.2 years. The sub-study had similar distribution of gender, age, and disease duration (Table 1). There was no significant difference between the disease activity (DAS, SJC, TJC, and HAQ) and burden (TGSS) and between the full trial and the biomarkers sub-population. Neither was there any difference in the level of the acute-phase reactants, CRP, and ESR (Table 1). There was no significant difference in the biomarkers baseline levels of the treatment groups (Table 1).

Efficacy profile; dose-dependent treatment effect on the serum biomarker

Approximately 59% of the placebo–MTX, 40% of the TCZ4 + MTX, and 28% of the TCZ8 + MTX patients were given rescue treatment, as described in the Patients and methods section. The following results show mean percentages of the baseline levels, and the *p*-values are derived from comparisons with the placebo–MTX group. Generally, the first infusion of both TCZ4 and TCZ8 induced a peak reduction from baseline at week 2 in serum levels of CRP, CRPM, C2M, C3M, and VICM and a peak increase in serum CTx and OC (Fig. 1).

Serum CRP was suppressed completely by TCZ8+MTX ($p < 0.0001$), to 39% ($p < 0.0001$) by TCZ4 + MTX and to 85% by placebo + MTX at week 16 (Fig. 1A). Serum CRPM, reflecting MMP-degraded CRP, was significantly ($p < 0.0001$) reduced to

Table 1
Summary of baseline demographics and disease activity in the biomarkers sub-study

	Full trial average [18,19]	PBO + MTX	TCZ4 + MTX	TCZ8 + MTX
N	1190	255	241	244
Female (%)	83	83	86	80
Age, mean ± SD years	52.0	51.6 ± 12.6	52.3 ± 13.1	53.8 ± 10.9
Disease duration, mean ± SD years	9.2	9.3 ± 8.2	10.3 ± 8.3	9.9 ± 8.7
DAS28, mean ± SD	6.5	6.5 ± 1.0	6.5 ± 0.9	6.5 ± 0.9
Swollen joint count (SJC), mean ± SD	17	16 ± 9	17 ± 10	16 ± 9
Tender joint count (TJC), mean ± SD	28	28 ± 16	27 ± 14	28 ± 15
HAQ score, mean ± SD	1.5	1.5 ± 0.6	1.5 ± 0.7	1.5 ± 0.6
Total Genant-modified Sharp score (TGSS), mean ± SD	28.7	27.9 ± 32.3	28.4 ± 28.5	28.2 ± 29.9
ESR, mean ± SD mm/h	46.3	46.5 ± 23.3	44.6 ± 23.4	44.2 ± 22.5
CRP, mean ± SD mg/dL	2.2	2.2 ± 2.5	2.0 ± 2.4	2.2 ± 2.6
CRPM, mean ± SD nmol/L		17.7 ± 9.4	16.6 ± 7.1	16.9 ± 8.8
C2M, mean ± SD nmol/L		0.56 ± 0.20	0.55 ± 0.17	0.55 ± 0.20
C3M, mean ± SD nmol/L		45.2 ± 23.4	43.8 ± 25.4	40.5 ± 17.9
VICM, mean ± SD nmol/L		10.4 ± 16.2	16.1 ± 50.5	20.7 ± 72.8
MMP3, mean ± SD mg/L		50.9 ± 50.1	48.3 ± 43.4	64.1 ± 81.0
CTx, mean ± SD ng/mL		0.43 ± 0.20	0.40 ± 0.19	0.41 ± 0.21
Osteocalcin, mean ± SD ng/mL		22.1 ± 14.2	21.8 ± 13.5	21.2 ± 13.2

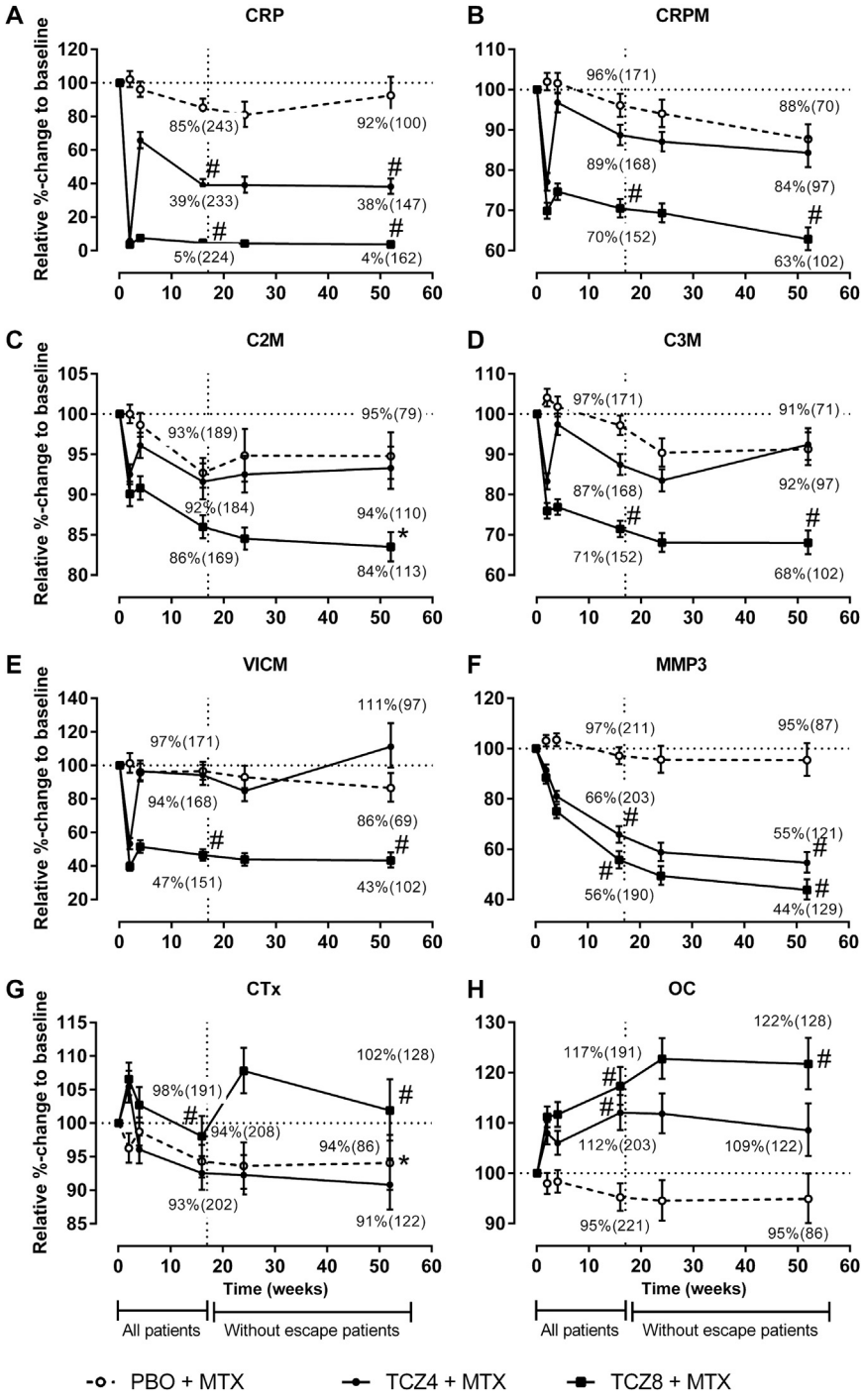

Fig. 1. Dose-dependent treatment biomarker profiles. Serum biomarkers were measured at baseline, 2, 4, 16, 24, and 52 weeks in the three treatment groups. (A) Normal serum CRP; (B) serum CRPM and CRP degraded by proteases; (C) serum C2M and cartilage degradation; (D) serum C3M and synovial turnover; (E) serum VICM, and citrullinated- and proteases-degraded vimentin; (F) serum MMP3 and circulating levels of MMP3; (G) serum CTx and bone resorption; and (H) serum OC and bone formation. The horizontal line gives the baseline level, and the vertical line gives the time of rescue treatment. The level of the biomarkers and the number of patients in each treatment group is indicated for week 16 (prior to rescue) and week 52 (end point). Data are shown as mean ± SEM bars and significance levels as * for $p < 0.05$ and # for $p < 0.001$.

70% by TCZ8 + MTX at week 16. TCZ4 + MTX reduced the level to 89% and placebo + MTX to 96%; however, there was no significant difference between the two TCZ-treated groups and the placebo group (Fig. 1B). After the escape point at week 16, serum CRPM continued to fall in all three groups reaching 88%, 84%, and 63% of baseline levels at week 52 (Fig. 1B). Thus, there was no difference between the TCZ4 + MTX- and placebo + MTX-treated groups. Serum C2M, which reflects cartilage degradation, was reduced to 93% of baseline levels by placebo + MTX, 92% by TCZ4 + MTX, and 86% ($p < 0.01$) by TCZ8 + MTX at week 16 (Fig. 1C). After the escape point, the level of C2M dropped an additional 2% in the TCZ8 + MTX group at week 52. Thus, TCZ4 did not prevent release of C2M any more than placebo + MTX. Serum C3M, reflecting synovial turnover, displayed a similar pattern as serum C2M. Serum C3M was reduced to 97% of baseline levels by placebo + MTX, to 87% by TCZ4 + MTX, and to 71% ($p < 0.0001$) for TCZ8 at week 16 (Fig. 1D). TCZ8 + MTX induced a further decrease in C3M, reaching 68% of baseline levels at week 52, whereas the slightly

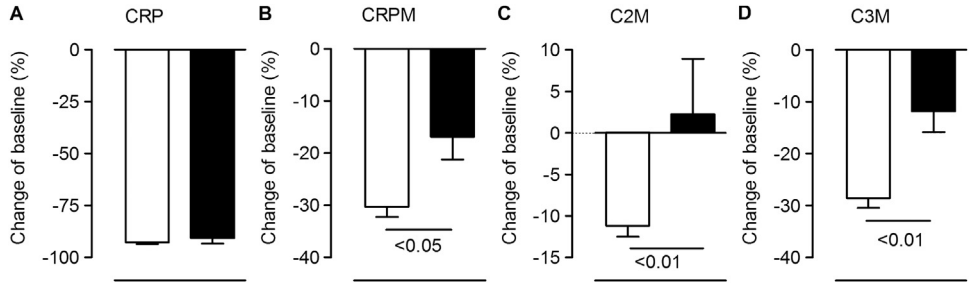

Fig. 2. The difference in biomarker levels at week 4 between early responders (ER, white bars) and early non-responders (ENR, black bars) after the first doses of TCZ8 + MTX. (A) Normal serum CRP; (B) serum CRPM and CRP degraded by proteases; (C) serum C2M, indicating cartilage degradation; and (D) serum C3M, indicating synovial turnover. Data are shown as the change from baseline giving the mean with SEM error bars. Significance levels at $p < 0.05$ and $p < 0.01$ are shown.

decreased level in the TCZ4 + MTX group could not be maintained. It reached 92% at week 52, which was a similar outcome for the placebo + MTX group (Fig. 1D)

Serum VICM, which measures the release of citrullinated vimentin fragments, was reduced to approximately 47% of baseline ($p < 0.0001$) by TCZ8 + MTX at week 16, whereas TCZ4 + MTX and placebo + MTX showed similar patterns to each other reaching 94% and 97% of baseline levels (Fig. 1E). Mean serum VICM dropped further in the TCZ8 + MTX group to 43% of baseline at week 52, but it increased in the TCZ4 + MTX group. However, the variation in VICM levels between patients on TCZ4 + MTX increased markedly at the end of the year, being 86% in the placebo + MTX group at week 52.

TCZ4 + MTX and TCZ8 + MTX were able to reduce the serum MMP3 to 66% and 56% at week 16 and to 55% and 44% at week 52, respectively. Placebo + MTX had no marked effect on MMP3 levels (Fig. 1F).

Serum CTx, a measure of bone resorption, was increased by TCZ8 + MTX, but only significantly ($p < 0.05$) at week 24 compared with placebo + MTX. TCZ4 + MTX and placebo + MTX showed a slight reduction from baseline levels (Fig. 1G). Bone formation, measured by serum OC, was significantly increased by TCZ4 + MTX to 112% ($p < 0.001$) and 109% (ns) and by TCZ8 + MTX to 117% ($p < 0.0001$) and 122% ($p < 0.0001$) at weeks 16 and 52, respectively, whereas placebo + MTX reduced the level of serum OC to 95% of baseline at both time points (Fig. 1H).

Odds ratio for early response to TCZ + MTX

Patients, who did not achieve a 20% reduction in SJC and TJC at week 16 and were given rescue therapy, were designated as early non-responders (ENR). The remaining patients were designated as early responders (ER).

The optimal biomarker cut-offs, determined by ROCs for the study population as a whole and for each individual treatment arm, are shown in Table 2. The change in CRP at week 4 was significantly predictive of response to treatment (OR 2.5, $p < 0.0001$). Change in CRP level was not predictive of response to placebo + MTX (OR 1.8, ns). Changes in CRP had some predictive value in the TCZ4 + MTX group (OR 2.2, $p < 0.05$), but not in the TCZ8 + MTX group (Table 2). The cut-off for optimal CRP levels in the TCZ8 + MTX group was approximately 92% (Fig. 2A), showing that both ER and ENR patients had suppressed CRP levels. The change in CRPM was predictive of a general response (OR 2.1, $p < 0.001$). The change was of borderline significance ($p < 0.05$) for the response to TCZ4 + MTX. The change in serum C2M as an effect of TCZ8 + MTX had an OR of 5.8 ($p < 0.001$) for predicting response, whereas no such thing was seen in the two other treatment arms. A change in C3M was strongly predictive of early improvement to TCZ8 + MTX with an OR of 9.6 ($p < 0.001$). Thus, CRPM, C2M, and C3M were the only three markers of the tested panel that were predictive of early response and then, only to TCZ8 + MTX treatment. The levels of these three biomarkers and CRP in the TCZ8+MTX group are shown in Fig. 2. The level of serum CRP was inhibited to the same extent in both ENRs and ERs (Fig. 2A). Serum CRPM was suppressed by TCZ8 + MTX by 30% for the ER group and by 16% for the ENR group—a difference that was significantly different ($p < 0.05$). Serum C2M was reduced by 12% in the TCZ8 + MTX ER group, whereas the level was slightly increased in ENR (Fig. 2C). Serum C3M was significantly suppressed in the responder group compared to the ENR (Fig. 2D).

Table 2
Odds ratios for early response to treatment measured by the change in the biomarkers from baseline to week 4

		All	PLACEBO + MTX	TCZ4 + MTX	TCZ8 + MTX
CRP	OR (95%CI)	2.5 (1.8–3.4)	1.8 (1.0–3.3)	2.2 (1.3–4.2)	1.6 (0.8–3.1)
	p Value	<0.0001	0.059	0.02	0.18
	Cut-off (%)	62.7	155	62.4	7.89
	AUC	0.62 (0.58–0.65)	0.50 (0.45–0.55)	0.58 (0.51–0.64)	0.55 (0.48–0.61)
CRPM	OR (95%CI)	2.1 (1.4–3.0)	2.2 (1.1–4.1)	2.0 (1.0–43.9)	3.5 (1.5–8.0)
	P value	0.0001	0.020	0.047	0.004
	Cut-off (%)	101	111	101	88.9
	AUC	0.59 (0.55–0.63)	0.51 (0.44–0.58)	0.53 (0.46–0.61)	0.64 (0.56–0.71)
C2M	OR (95%CI)	1.9 (1.3–2.7)	1.4 (0.7–2.7)	1.8 (0.9–3.5)	5.8 (2.2–15)
	p Value	0.0012	0.29	0.11	0.0003
	Cut-off (%)	92.6	88.2	106	92.6
	AUC	0.58 (0.54–0.62)	0.51 (0.44–0.58)	0.53 (0.46–0.60)	0.69 (0.61–0.75)
C3M	OR (95%CI)	6.2 (3.3–11)	1.7 (0.9–3.0)	2.5 (0.8–7.5)	8.1 (2.3–28)
	P value	<0.0001	0.75	0.11	0.001
	Cut-off (%)	71.2	100	127	71.6
	AUC	0.60 (0.56–0.64)	0.54 (0.47–0.62)	0.52 (0.45–0.59)	0.68 (0.60–0.75)

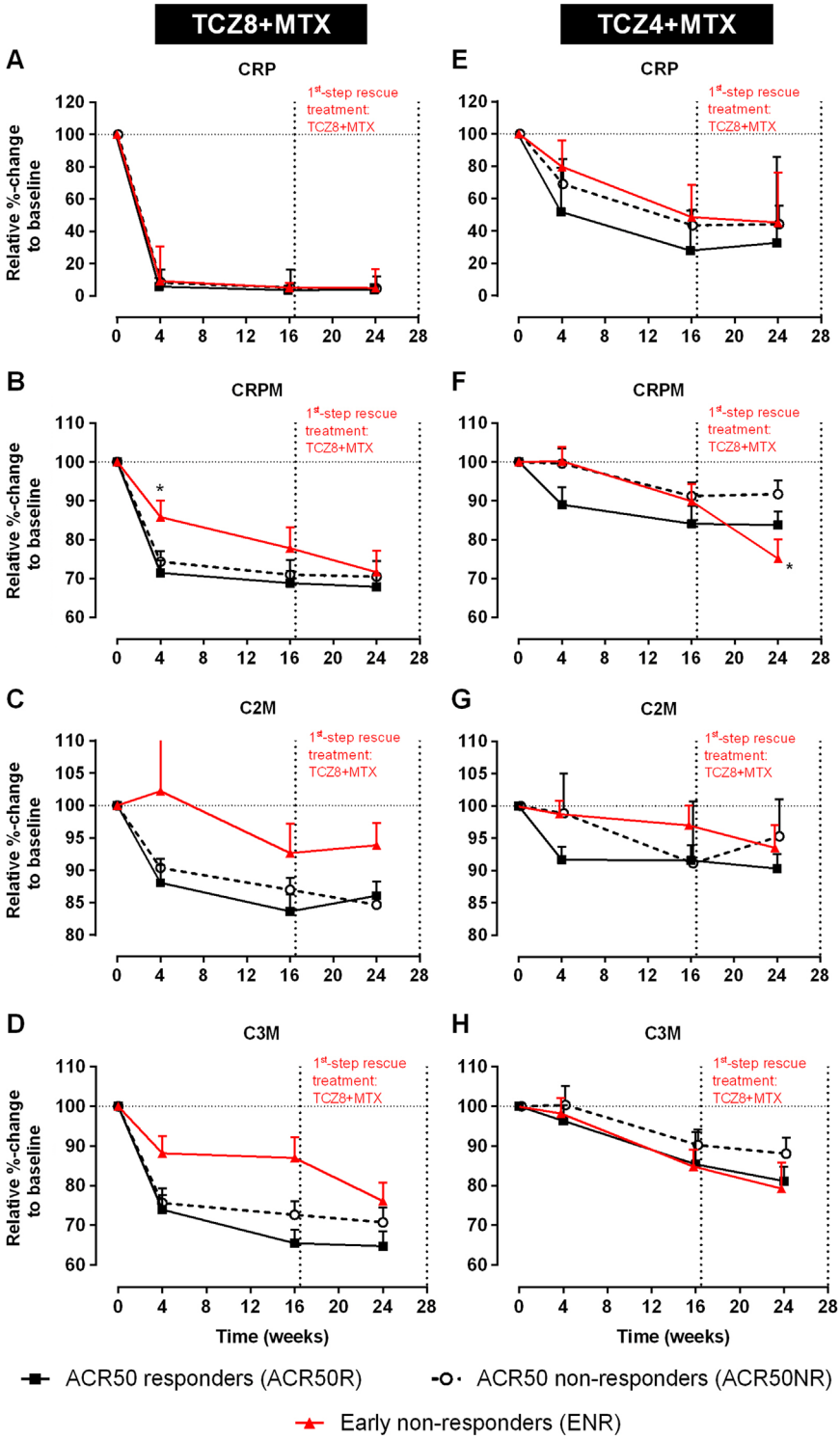

Fig. 3. The biomarker profile of patients classified as ACR50 responders (black squares), non-responders (open circles), or the ENR (gray triangles) from baseline to week 52. (A) Serum CRP levels in response to TCZ8. (B) Serum CRPM levels in response to TCZ8. (C) Serum C2M levels in response to TCZ8. (D) Serum C3M levels in response to TCZ8. (E) Serum CRP levels in response to TCZ4. (F) CRPM levels in response to TCZ4. (G) Serum C2M levels in response to TCZ4. (H) Serum C3M levels in response to TCZ4. Data are shown as mean with SEMs.

Difference in serum biomarker profiles of ACR50 responders and non-responders

An analysis of ENRs, and the 52-week ACR50 responders (ACR50R) and non-responders (ACR50NR), showed that serum level of CRP was completely inhibited by TCZ8 + MTX in all three groups (Fig. 3A). Serum CRPM was decreased in response to TCZ8 + MTX in the ACR50R and the ACR50NR groups to approximately 74% and 72% at week 4, and to 71% and 68% at week 24 (Fig. 3B). Serum CRPM levels also decreased in the ENR group, but not to the same extent as in the other groups. After the first-step rescue treatment was administered to the ENR group at week 16, the level

of CRPM decreased to the level of the ACR50NR group (Fig. 3B). The cartilage degradation marker, C2M, and synovial turnover marker, C3M, were initially inhibited by TCZ8 + MTX to a greater extent in the ACR50R group than in the ACR50NR group (Fig. 3C and D). The ENR group displayed a markedly different profile to the other two groups (Fig. 3C and D). C3M levels decreased markedly after first-step rescue treatment.

Serum CRP levels were inhibited the most in the ACR50R group receiving TCZ4 + MTX. However, the between-patient variation was large, indicating that some patients had significantly inhibited levels while others did not. The two non-responder groups followed a similar pattern to each other (Fig. 3E). CRPM was significantly inhibited in the ACR50R group compared to the ACR50NR (Fig. 3F). The level of CRPM slightly decreased in a similar fashion in the ENR and ACR50NR until the first-step rescue point, after which the level decreased further in the ENR group (Fig. 3F). Serum C2M was significantly lower in the ACR50R than in the two non-responders groups prior to the first rescue point (Fig. 3G). There was no significant difference in the level of serum C3M in either non-responder group (Fig. 3H), although the ACR50NR group seemed to have a less inhibited level of C3M.

Discussion

This 1-year sub-study of the LITHE trial investigated eight biomarkers of joint destruction and inflammation. The novel biomarkers are all neo-epitopes released during tissue processing, and so are measures of pathological events in inflamed tissue [22]. We investigated the biomarker profile in response to 4- or 8-mg/kg TCZ treatment, or placebo, combined with MTX. We demonstrated that there was a significant difference between the two active doses and the placebo group for specific markers of tissue inflammation and cartilage turnover. Intact CRP levels were inhibited by both doses of TCZ, as expected, whereas cartilage degradation, as measured by C2M, and synovial inflammation, as measured by serum CRPM and C3M, were only significantly inhibited by TCZ8 + MTX. These results indicate that TCZ4 + MTX indeed had anti-inflammatory activity on the systemic level, but this did not convert into a protective effect on joint tissue. Our analysis of the ability of these biomarkers to discriminate between early responders and non-responders showed that patients with a significant decrease in serum levels of CRPM, C2M, and C3M, but not intact CRP, had a significantly higher OR for response to TCZ8 + MTX, but not to TCZ4 + MTX. These results indicate that the individual biomarkers may possess predictive properties for TCZ response. Lastly, we found that the dose-dependent profiles of these markers were significantly different between ACR50 responders and non-responders, as well as early non-responders (escape patients). These last results suggest that the neo-epitope biomarkers can be used to identify responders and non-responders at an early time point

The biomarkers were time-dependently attenuated by the TCZ administration, which may suggest a continued clinical benefit of the intervention. In particular, the CRPM marker, in contrast to traditional CRP, showed time-dependent resolution. CRP, which is one of the main clinical targets of anti-IL6R treatment, is produced in the liver in response to increased levels of IL-6 [15,23,24]. TCZ8 completely inhibited the level of CRP, while TCZ4 only inhibited the level of CRP by 50%. The limited effect of MTX on CRP levels in the current study compared with previous studies [25] was somewhat predictive of MTX response, with an OR of 6.5 and an optimal cut-off of 55%. Patients who maintained their CRP at less than 55% of baseline were likely to be early responders. However, CRP did not predict clinical response to TCZ. In contrast, serum CRPM was actually predictive of clinical response to TCZ. The ORs for prediction of early clinical response to TCZ8 and TCZ4 were 4 and 2, respectively. Interestingly, the separation between ACR50 responders and non-responders to TCZ8 became more pronounced over time. An explanation for this could be that as CRP production was inhibited by anti-IL-6 treatment, the deposition of CRP in the inflamed tissue was attenuated and consequently the cleavage of it was likewise inhibited after a time lag. This is illustrated in the differences in the profiles of CRP and CRPM in responders and non-responders. While CRP was completely inhibited by TCZ8 in ACR50 responders and non-responders, the decrease in CRPM levels was significantly greater in the ACR50 responder group than non-responders. The continued decrease in CRPM over the year may have been the result of previously deposited CRP being slowly released and the joint is rebalancing toward a healthier phenotype.

Cartilage degradation and synovial turnover, measured by serum C2M and C3M, were decreased by TCZ8, but not by TCZ4. This suggests a marked difference in the physiological response to the two doses. Changes in serum VICM showed the similar dose–time profile. MMP3, which is believed to be one of the MMPs involved in tissue degradation, was lowered to the same extent for both doses of TCZ. MMP3 is however not the only MMP involved in joint tissue degradation [26]. Several MMPs have been found to be up-regulated in RA [27,28], many of which are directly regulated by IL-6 and CRP [24,29]. This may be one explanation to why there is a disconnection between the change in the MMP-derived tissue biomarkers (i.e., C2M, C3M, VICM, and CRPM) and MMP3. TCZ + MTX induced a rapid and dose-dependent reduction in MMP3 with a significantly larger reduction with the 8-mg/kg dose. This result suggests that TCZ 8 mg/kg in combination with MTX is more effective than the 4-mg/kg dose in decreasing cartilage turnover and degradation, and that TCZ limits joint tissue inflammation.

Our results confirm a similar biomarker analysis performed for the RADIATE study, examining the effects of TCZ on bone and inflammation [16]. TCZ added to MTX induced a modest, but significant and dose-dependent increase in the systemic bone formation marker in the RADIATE analysis [16], as it did in the current study, with maintenance of bone resorption. These results provide evidence that TCZ, especially at the dose of 8 mg/kg, has a positive effect on bone balance, which in untreated patients [22,30–32] is imbalanced, leading to continued bone loss. This notion was supported by Terpos et al. [33], who observed a disruption of the bone balance with 8-mg/kg TCZ by measurement of bone remodeling markers of the wnt pathway. Although the consequence of the effect of TCZ on bone mineral density and fragility fracture is difficult to predict, the magnitude of the observed changes is greater than the physiological within-patient variability and is similar to those observed with strontium ranelate, an effective therapy in postmenopausal osteoporosis [34,35]. The effect of IL-6 receptor inhibition on bone remodeling in humans should be further examined.

The ACR50 non-responders in the TCZ4 group might have had an initial inhibition of joint inflammation, providing initial and sufficient clinical benefit to avoid the ACR50 non-responders from being given rescue treatment. An important question is whether they would have benefited from dose-escalation. The patients treated with TCZ4, who did not respond to treatment at week 16 (and were thus deemed as escape patients), were escalated to TCZ8. Serum CRP levels for these patients (ENR) were similar to those of the ACR50 non-responder group. However, the level of CRPM dropped markedly after escalation of the dose. A similar pattern was observed in markers of cartilage degradation: the level of C2M dropped to the level of the ACR50R group after escalating the dose, and continued to drop after the second rescue point. Serum levels of C3M were likewise markedly reduced by the

escalating dose. It is important to note that the patients who did not pass the second escape point evaluation left the study. Thus, the patients remaining in the escape group were patients who had some level of clinical response. These results indicate that a subset of patients actually benefited from increased dose of TCZ and that the applied biomarkers probably could assist in the identification of patients who would benefit from an up-scaled dose.

The main limitation of the study is that radiographic evidence showed only a very low rate of progression, especially in the TCZ treatment groups, which impaired the power to detect significant associations and created large confidence intervals for the odds ratios. Baseline correlations between the biomarkers and clinical characteristics were performed; however, it would be of significant interest to investigate whether these same markers would be prognostic of disease progression.

We have in this study measured serum levels of end products of tissue destruction, which clearly provide additional value than measurement of individual proteases and cytokines regulating tissue destruction. RA is heavily regulated and driven by pro-inflammatory cytokines, such as IL-1, IL-6, IL-17, and TNFα. Measurement of the level of these cytokines as predictive markers has yet to be supported. While the variation in the levels of the individual cytokines and consequent signaling pathways may be large, the common end products are tissue destruction by cleavage of joint-specific proteins mediated by the increase in the proteases [4]. Such biomarkers measure the burden of disease by, for example, indicating how much cartilage is being lost, and the efficacy of the intervention directly on the tissue in question. The same biomarkers are being investigated in other rheumatic diseases, such as osteoarthritis (OA) and ankylosing spondylitis (AS). In OA, serum and urinary levels of cartilage products were correlated with JSN [6,36–39]. In AS, both biomarkers of cartilage and synovial turnover were increased in patients with a high burden of disease as compared to patients with low burden and controls [12,40].

Conclusion

In conclusion, we have identified biomarkers that indicate responders to treatment and that reflect joint deterioration and inflammation. These markers are closely associated with the pathology of the joints, and may thus be considered as more true biomarkers of efficacy, and thus better tools for monitoring and understanding drug efficacy and mode of actions, than standard markers. The marker panel we identified may be useful for identifying the patients who would benefit the most from TCZ treatment and those who would benefit from escalating the dose from 4 to 8 mg/kg. Whether these profiles are transferrable to other indications or interventions needs to be tested further.

Competing interests

Anne C. Bay-Jensen, Inger Byrjalsen, and Morten A. Karsdal are full-time employees at Nordic Bioscience. Claus Christiansen and Morten A. Karsdal hold stocks in Nordic Bioscience. Nordic Bioscience is a privately owned, small–medium size enterprise (SME) partly focused on the development of biomarkers for rheumatic and fibrotic diseases. None of the authors received fees, bonuses, or other benefits for the work described in the manuscript. Philippe Vergnoud is a full-time employee of Synarc laboratories. Adam Platt has no financial interest to declare. The biomarker part of the study was supported by the Danish Research Foundation.

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References

[1] Schett G, Hayer S, Zwerina J, Redlich K, Smolen JS. Mechanisms of disease: the link between RANKL and arthritic bone disease. Nat Clin Pract Rheumatol 2005;1(1):47–54.
[2] Diarra D, Stolina M, Polzer K, Zwerina J, Ominsky MS, Dwyer D, et al. Dickkopf-1 is a master regulator of joint remodeling. Nat Med 2007;13(2):156–63.
[3] Yazici Y, Regens AL. Promising new treatments for rheumatoid arthritis—the kinase inhibitors. Bull NYU Hosp Jt Dis 2011;69(3):233–7.
[4] Karsdal MA, Delvin E, Christiansen C. Protein fingerprints—relying on and understanding the information of serological protein measurements. Clin Biochem 2011;44(16):1278–9.
[5] Karsdal MA, Henriksen K, Leeming DJ, Mitchell P, Duffin K, Barascuk N, 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(3):181–202.
[6] Bay-Jensen AC, Liu Q, Byrjalsen I, Li Y, Wang J, Pedersen C, 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(5–6):423–9.
[7] Garnero P, Conrozier T, Christgau S, Mathieu P, Delmas PD, Vignon E. Urinary type II collagen C-telopeptide levels are increased in patients with rapidly destructive hip osteoarthritis. Ann Rheum Dis 2003;62(10):939–43.
[8] Karsdal MA, Byrjalsen I, Bay-Jensen AC, Henriksen K, Riis BJ, Christiansen C. Biochemical markers identify influences on bone and cartilage degradation in osteoarthritis—the effect of sex, Kellgren-Lawrence (KL) score, body mass index (BMI), oral salmon calcitonin (sCT) treatment and diurnal variation. BMC Musculoskelet Disord 2010;11:125.
[9] Syversen SW, Haavardsholm EA, Boyesen P, Goll GL, Okkenhaug C, Gaarder PI, et al. Biomarkers in early rheumatoid arthritis: longitudinal associations with inflammation and joint destruction measured by magnetic resonance imaging and conventional radiographs. Ann Rheum Dis 2010;69(5):845–50.
[10] Nemirovskiy OV, Dufield DR, Sunyer T, Aggarwal P, Welsch DJ, Mathews WR. Discovery and development of a type II collagen neoepitope (TIINE) biomarker for matrix metalloproteinase activity: from in vitro to in vivo. Anal Biochem 2007;361(1):93–101.
[11] Barascuk N, Veidal SS, Larsen L, Larsen DV, Larsen MR, Wang J, 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 neo-epitope of type III collagen. Clin Biochem, http://dx.doi.org/10.1016/j.clinbiochem.2010.03.012.
[12] Bay-Jensen AC, Leeming DJ, Kleyer A, Veidal SS, Schett G, Karsdal MA. Ankylosing spondylitis is characterized by an increased turnover of several different metalloproteinase-derived collagen species: a cross-sectional study. Rheumatol Int 2012;32(11):3565–72.
[13] Crowson CS, Rahman MU, Matteson EL. Which measure of inflammation to use? A comparison of erythrocyte sedimentation rate and C-reactive protein measurements from randomized clinical trials of golimumab in rheumatoid arthritis J Rheumatol 2009;36(8):1606–10.
[14] Skjot-Arkil H, Schett G, Zhang C, Larsen DV, Wang Y, Zheng Q, et al. Investigation of two novel biochemical markers of inflammation, matrix metalloproteinase and cathepsin generated fragments of C-reactive protein, in patients with ankylosing spondylitis. Clin Exp Rheumatol 2012;30(3):371–9.
[15] Kishimoto T. Interleukin-6: discovery of a pleiotropic cytokine. Arthritis Res Ther 2006;8(Suppl 2):S2.
[16] Karsdal MA, Schett G, Emery P, Harari O, Byrjalsen I, Kenwright A, et al. IL-6 receptor inhibition positively modulates bone balance in rheumatoid arthritis patients with an inadequate response to anti-tumor necrosis factor therapy: biochemical marker analysis of bone metabolism in the tocilizumab RADIATE study (NCT00106522). Semin Arthritis Rheum 2012;42(3):131–9.
[17] Garnero P, Thompson E, Woodworth T, Smolen JS. Rapid and sustained improvement in bone and cartilage turnover markers with the anti-interleukin-6 receptor inhibitor tocilizumab plus methotrexate in rheumatoid arthritis patients with an inadequate response to methotrexate: results from a substudy of the multicenter double-blind, placebo-controlled trial of tocilizumab in inadequate responders to methotrexate alone. Arthritis Rheum 2010;62(1):33–43.

[18] Smolen JS, Avila JC, Aletaha D. Tocilizumab inhibits progression of joint damage in rheumatoid arthritis irrespective of its anti-inflammatory effects: disassociation of the link between inflammation and destruction. Ann Rheum Dis 2012;71(5):687–93.
[19] Kremer JM, Blanco R, Brzosko M, Burgos-Vargas R, Halland AM, Vernon E, et al. Tocilizumab inhibits structural joint damage in rheumatoid arthritis patients with inadequate responses to methotrexate: results from the double-blind treatment phase of a randomized placebo-controlled trial of tocilizumab safety and prevention of structural joint damage at one year. Arthritis Rheum 2011;63(3):609–21.
[20] Bay-Jensen AC, Karsdal MA, Vassiliadis E, Wichuk S, Marcher-Mikkelsen K, Lories R, et al. Circulating citrullinated vimentin fragments reflect disease burden in ankylosing spondylitis and have prognostic capacity for radiographic progression. Arthritis Rheum 2013;65(4):972–80.
[21] Claudon A, Vergnaud P, Valverde C, Mayr A, Klause U, Garnero P. New automated multiplex assay for bone turnover markers in osteoporosis. Clin Chem 2008;54(9):1554–63.
[22] Karsdal MA, Woodworth T, Henriksen K, Maksymowych WP, Genant H, Vergnaud P, et al. Biochemical markers of ongoing joint damage in rheumatoid arthritis—current and future applications, limitations and opportunities. Arthritis Res Ther 2011;13(2):215.
[23] Hurlimann J, Thorbecke GJ, Hochwald GM. The liver as the site of C-reactive protein formation. J Exp Med 1966;123(2):365–78.
[24] Marnell L, Mold C, Du Clos TW. C-reactive protein: ligands, receptors and role in inflammation. Clin Immunol 2005;117(2):104–11.
[25] Jones G, Sebba A, Gu J, Lowenstein MB, Calvo A, Gomez-Reino JJ, et al. Comparison of tocilizumab monotherapy versus methotrexate monotherapy in patients with moderate to severe rheumatoid arthritis: the AMBITION study. Ann Rheum Dis 2010;69(1):88–96.
[26] Burrage PS, Mix KS, Brinckerhoff CE. Matrix metalloproteinases: role in arthritis. Front Biosci 2006;11:529–43.
[27] Birkedal-Hansen H. Proteolytic remodeling of extracellular matrix. Curr Opin Cell Biol 1995;7(5):728–35.
[28] Bode W, Maskos K. Structural basis of the matrix metalloproteinases and their physiological inhibitors, the tissue inhibitors of metalloproteinases. Biol Chem 2003;384(6):863–72.
[29] Chakraborti S, Mandal M, Das S, Mandal A, Chakraborti T. Regulation of matrix metalloproteinases: an overview. Mol Cell Biochem 2003;253:269–85.
[30] Landewe RB, Geusens P, van der Heijde DM, Boers M, van der LS, Garnero P. Arthritis instantaneously causes collagen type-I and type-II degradation in patients with early rheumatoid arthritis: a longitudinal analysis. Ann Rheum Dis 2006;65(1):40–4. Epub 2005, Aug 26.
[31] Garnero P, Gineyts E, Christgau S, Finck B, Delmas PD. Association of baseline levels of urinary glucosyl-galactosyl-pyridinoline and type II collagen C-telopeptide with progression of joint destruction in patients with early rheumatoid arthritis. Arthritis Rheum 2002;46(1):21–30.
[32] Lories R. The balance of tissue repair and remodeling in chronic arthritis. Nat Rev Rheumatol 2011;7(12):700–7.
[33] Terpos E, Fragiadaki K, Konsta M, Bratengeier C, Papatheodorou A, Sfikakis PP. Early effects of IL-6 receptor inhibition on bone homeostasis: a pilot study in women with rheumatoid arthritis. Clin Exp Rheumatol 2011;29(6):921–5.
[34] Delmas PD. Clinical effects of strontium ranelate in women with postmenopausal osteoporosis. Osteoporos Int 2005;16(Suppl. 1):S16–9.
[35] Meunier PJ, Roux C, Seeman E, Ortolani S, Badurski JE, Spector TD, et al. The effects of strontium ranelate on the risk of vertebral fracture in women with postmenopausal osteoporosis. N Engl J Med 2004;350(5):459–68.
[36] Bay-Jensen AC, Andersen TL, Charni-Ben TN, Kristensen PW, Kjaersgaard-Andersen P, Sandell L, et al. Biochemical markers of type II collagen breakdown and synthesis are positioned at specific sites in human osteoarthritic knee cartilage. Osteoarthritis Cartilage 2008;16(5):615–23.
[37] Bay-Jensen AC, Sondergaard BC, Christiansen C, Karsdal MA, Madsen SH, Qvist P. Biochemical markers of joint tissue turnover. Assay Drug Dev Technol 2010;8(1):118–24.
[38] Dam EB, Loog M, Christiansen C, Byrjalsen I, Folkesson J, Nielsen M, et al. Identification of progressors in osteoarthritis by combining biochemical and MRI-based markers. Arthritis Res Ther 2009;11(4):R115.
[39] Dam EB, Byrjalsen I, Karsdal MA, Qvist P, Christiansen C. Increased urinary excretion of C-telopeptides of type II collagen (CTX-II) predicts cartilage loss over 21 months by MRI. Osteoarthritis Cartilage 2009;17(3):384–9.
[40] Maksymowych WP, Landewe R, Conner-Spady B, Dougados M, Mielants H, van der TH, et al. Serum matrix metalloproteinase 3 is an independent predictor of structural damage progression in patients with ankylosing spondylitis. Arthritis Rheum 2007;56(6):1846–53.