

Connective tissue remodelling is differently modulated by Tocilizumab versus Methotrexate monotherapy in patients with early Rheumatoid Arthritis: the AMBITION study

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Abstract

Objective: Associations between Rheumatoid Arthritis (RA) and effect of treatment at the tissue levels are poorly understood. We investigated the scope of released extracellular matrix (ECM) metabolites as a consequence of tissue remodeling in patients treated with Methotrexate (MTX) and Tocilizumab (TCZ) compared to placebo.

Methods: Tissue metabolites from 387 RA patients treated with either TCZ (8 mg/kg) or MTX monotherapy (7.5–20 mg/kg) were measured at baseline and 8-weeks sera by validated ELISA assays. The levels of collagen biomarkers (C1M, C2M, C3M and C4M) together with C-reactive protein (CRP) and CRP metabolite (CRPM) were investigated. Baseline levels of biomarkers have been compared with 72 age and gender matched healthy controls. Comparison between treatment and response groups were done by ANCOVA, Spearman's correlation and Logistic regression adjusted for age, gender, BMI and disease duration.

Results: C1M and C3M, were significantly ($P < 0.05$) inhibited by TCZ and C3M by MTX ($P < 0.01$) compared to placebo. C1M and C3M inhibition with TCZ was respectively 23% and 16% greater than that of MTX ($P < 0.01$ and $P < 0.0001$). C4M was inhibited by TCZ and MTX, but the effect of TCZ was 22% greater than MTX ($P < 0.0001$). TCZ and MTX had minimal effect on C2M levels. MTX had no effect on CRP and CRPM, whereas TCZ reduced their levels to 69% and 27% from baseline. Investigated biomarkers revealed a significant ($P < 0.05$) differences in biomarker profiles of MTX ACR50 treatment responders and non-responders. Change to week 8 in levels of C3M, C4M, CRP and CRPM in MTX patients correlated significantly ($\rho = 0.41$ to 0.18 , $P < 0.0001$ to 0.039) with change in disease activity (DAS28) at week 8, 16 and 24, whereas only CRP in TCZ patients ($\rho = 0.32$ to 0.21 , $P < 0.0001$ to 0.01).

Conclusion: Patients receiving TCZ treatment for 8 weeks had higher suppression of tissue remodeling and inflammatory biomarkers over patients treated with MTX. Measured biomarkers enabled for a discrimination of biomarker profiles of ACR50 treatment responding patients and identification of those who benefit at the early time-point. Week 8 change in levels of C3M, C4M, CRP and CRPM significantly predicted clinical response to treatment and correlated with DAS28 at all time-points.

1. Introduction

Rheumatoid Arthritis (RA) is a complex autoimmune, inflammatory disease, which primarily affects synovial joints and causes local damage along with systemic manifestations [1–2]. Its prevalent in 0.5–1% of the world's population. The disease has a progressive course leading to permanent disability and is therefore associated with extensive personal, social, and economic impacts. Despite decades of research into this disease, the etiology of RA is still not completely understood, but is known to be dependent on both genetic susceptibility and environmental factors [3].

The introduction of disease-modifying anti-rheumatic drugs (DMARDs) such as Methotrexate (MTX) has substantially changed the prognosis and improved everyday life of RA patients. MTX relieve disease

symptoms and remains the most commonly used disease-modifying anti-rheumatic drug [4–5]. However, therapies involving MTX often have inadequate response or intolerance, which calls for alternative treatments such as biological agents [6]. One of them is Tocilizumab (TCZ), a humanized anti-IL-6 receptor monoclonal antibody, which acts by blocking IL-6 signaling [7–9]. Elevated levels of IL-6 in synovial fluid and serum correlate with disease activity in patients with RA [10–11]. Inhibition of the IL-6 signaling by TCZ showed a protective effect on bone erosions by decreasing their volume in RA patients and significant reduction of swollen and tender joint counts with concurrent improvement in radiographic progression [12][9].

Key histological features of RA affected joints include hyperplasia and cellular infiltration of the synovium and systemic inflammation. [13]. The inflammation leads to accelerated remodeling of extracellular matrix (ECM) that can progress to bone erosion and cartilage destruction [14–15]. Destruction of connective tissue, especially the ECM of cartilage, bone, and soft tissues of the joint, is a hallmark of RA pathology [16].

Proteolytic enzymes expressed locally in the pathologically affected tissue, such as the matrix metalloproteinases (MMPs) and aggrecanases, have a role in the remodeling of the ECM.

In healthy connective tissues, physiologic expression of these enzymes is low, but increases considerably during the inflammation process in RA thereby leading to excessive ECM remodeling [17]. We have previously shown that MMP-3 was highly elevated in RA and treatment with TCZ significantly decreased MMP3 levels [18]. The consequence of MMPs upregulation is heightened ECM remodeling with the release of a range of protein specific degradation products (neo-epitopes) to circulation that can be used as biomarkers of tissue remodeling in joint diseases [19]. Former findings have shown that upregulated levels of MMP-degraded type I, II, III and IV collagen (C1M, C2M, C3M and C4M respectively) correlated with disease activity in RA patients and treatment with TCZ + MTX significantly suppressed the biomarkers dose-dependently [20–23]. C1M was shown not only to be associated with disease activity, but a useful tool for identification of RA patients with fast structural progression [22]. Gudmann et al., have shown that C4M together with C1M and C3M are biomarkers reflecting structural joint degradation in RA and their baseline elevated levels significantly correlates with disease activity score of 28 joints (DAS28) [23].

Another type of biomarkers are biomarkers that indicate an ongoing inflammatory process. C-reactive protein (CRP), a biomarker of systemic inflammation, is produced and released from the liver as an acute reactant during inflammation, whereas CRPM, an MMP-degraded fragment of CRP, is released from the inflamed tissue. CRP levels have shown predict clinical outcomes from treatment with TCZ in RA and many other inflammatory diseases [24]. It was reported that levels of CRPM correlate with RA activity at different disease stages and significantly downregulate together with collagen biomarkers after TCZ + MTX treatment [25][26][21]. Serological biomarkers of tissue remodeling and inflammation may give insight into treatment effect at the tissue level of different interventions giving an alternative approach to currently used methods such as symptomatic changes assessed by ACR-response or DAS28. In our study,

we investigated if tissue remodeling was differently modulated by TCZ and MTX monotherapies in RA patients by measuring tissue remodeling biomarkers.

2. Materials And Methods

2.1 Patients and study design

The AMBITION study (NCT00109408) was a 24-week phase 3 trial, double-blind, double-dummy, parallel-group, including 673 biological naive patients, randomised to either Tocilizumab (TCZ) monotherapy 8 mg/kg intravenously every 4 weeks or Methotrexate (MTX) monotherapy oral capsules every week (initial dose 7.5 mg and titrated to 15 mg at week 4 and to 20 mg at week 8) [27]. Placebo (PBO) patients received tocilizumab 8 mg/kg from week 8 until the end of the study. The current biomarker sub-study of AMBITION involved baseline and week 8 serum samples from a total number of 387 adult patients (> 18 years) with moderate to severe active RA (Fig. 1). The AMBITION study was approved by the ethics committee at each participating institution (in USA, Canada and Israel) and was conducted in accordance with the Principles of Good Clinical Practice and according to the Declaration of Helsinki. All patients included provided written, informed consent before inclusion in the study. Serum from 72 healthy controls included in the study was obtained from the Discovery Life Sciences, Inc. vendor with compliance to the Ethics Committee recommendations and all regulations, guidelines and best practices that meet or exceed the U.S. and international regulatory requirements.

2.2 Biochemical marker assays

Serum levels of type I [28], II [29], III [30], and IV [31] collagen degradation biomarkers together with CRP and its metabolite CRPM [25] were measured in serum by technically validated ELISAs developed by Nordic Bioscience (Herlev, Denmark). Biomarkers were measured according to manufacture instructions. A brief description of the protocol follows: Streptavidin-coated 96-well plates were coated with respective biotinylated antigens for 30 min at 20°C and subsequently washed 5 times in washing buffer. Appropriate controls, standard, and serum samples were added with subsequent addition of peroxidase conjugated antibody against the respective epitope.

The mixture was incubated depending of an assay for 1 hour at 20C (C3M, C4M, CRPM) or 20 hours (reC1M, C2M) at 4°C. Plates were subsequently washed 5 times with washing buffer. TMB substrate (3,3',5,5' – tetramethylbenzidine) was added and incubated for 15 min at 20°C and the reaction was stopped with 0.18 M sulphuric acid. A SpectraMax Microplate Reader (Molecular Devices Corporation, Sunnyvale, CA, USA) was used to read the absorbance at 450 nm with reference set to 650 nm. The concentrations were calculated by using a 4-parametric curve fit model. Biomarker results were approved under fulfilling acceptance requirements of an analytical run. This comprised the acceptance of three main criteria. Firstly, acceptance of the standard curve with all standards points resulting in <10% coefficient of variance (CV) and <15% of relative error (%RE) within the analytical measuring range. The exception from that criteria was that one standard point could be masked if CV > 10% or if %RE > 15%. Secondly, acceptance of the quality controls with all resulting in <15% CV for all QC specimens and within

the target range of mean \pm 20%, with the exception that one QC specimen was allowed to have CV > 15%. Lastly, acceptance of sample measurement for samples within the measurement range and <15% CV. Samples with a CV > 15% were re-analyzed.

2.3 Statistical analyses

The distribution of patient's baseline parameters among different treatment groups was assessed by one-way ANOVA t-test and presented in Table 1. Biomarker values that were above (ULOD) detection range were remeasured in dilution or excluded from the further analysis. The lower detection limit (LLOD) values were handled by setting the detection limit cutoff for confident determination of sample concentration and statistical differentiation from a blank at a 99% confidence level. Change in biomarker levels at week 8 in relation to baseline was tested by paired *t*-test for each treatment (Fig. 2). Comparison between treatment groups (TCZ, MTX and PBO) was examined by ANCOVA at baseline and week 8, adjusted for age, gender, BMI, and disease duration.

Table 1. Patients demographics and baseline characteristics of the AMBITION cohort, according to treatment group. Differences between treatment groups are presented by one-way ANOVA t-test.

Characteristics	Tocilizumab N = 164	Methotrexate N = 161	Placebo N = 62	P Value
Age (years), mean (SD)	50.7 (12.9)	51.3 (13.3)	50.5 (11.8)	ns
Female, n (%)	131 (80)	122 (76)	47 (76)	ns
Male, n (%)	33 (20)	39 (24)	15 (24)	ns
DAS28, mean (SD)	6.8 (0.9)	6.8 (0.9)	6.9 (0.9)	ns
BMI (SD)	28.1 (6.4)	27.4 (6.0)	29.1 (7.4)	ns
TJC, mean (SD)	32.6 (14.8)	32.1 (14.1)	35.4 (16.3)	ns
SJC, mean (SD)	19.4 (10.9)	19.9 (10.6)	23.5 (12.2)	P = 0.04
RADUR (SD)	6.7 (8.1)	6.6 (8.0)	7.4 (9.0)	ns
CRP (mg/dL), mean (SD)	3.2 (3.6)	3.1 (3.5)	2.5 (2.7)	ns
ESR (mm/h), mean (SD)	46.9 (26.1)	47.9 (24.1)	48.2 (22.4)	ns
HAQ-DI, mean (SD)	1.6 (0.6)	1.6 (0.6)	1.4 (0.5)	ns
Pain VAS 100 mm, mean (SD)	59.0 (22.4)	61.3 (20.7)	59.5 (24.4)	ns
Patient VAS 100 mm, mean (SD)	64.6 (22.0)	64.9 (19.4)	64.3 (22.5)	ns
Physician VAS 100 mm, mean (SD)	63.5 (16.1)	64.6 (16.3)	69.1 (17.9)	ns
Baseline C1M (ng/mL), mean (95% CI)	42.7 (37.5-47.8)	45.2 (39.4-50.9)	40.5 (34.5-46.5)	ns
Baseline C2M (ng/mL), mean (95% CI)	0.4 (0.4-0.5)	0.4 (0.4-0.5)	0.3 (0.3-0.4)	ns
Baseline C3M (ng/mL), mean (95% CI)	17.8 (16.5-19.2)	18.3 (17.1-19.5)	15.9 (13.8-17.9)	ns
Baseline C4M (ng/mL), mean (95% CI)	53.9 (49.3-58.4)	51 (47.2-54.8)	42.3 (36.5-48.1)	P = 0.02
Baseline CRPM (ng/mL), mean (95% CI)	21.9 (19.6-24.2)	20.8 (18.9-22.8)	18.5 (15.0-21.9)	ns
Baseline CRP (ng/mL), mean (95% CI)	3.2 (2.6-3.8)	3.1 (2.6-3.7)	2.5 (1.8 – 3.2)	ns

DAS28 28-Joint Disease Activity Score; **TJC** tender joint count; **SJC** swollen joint count; **CRP** C-reactive protein; **ESR** erythrocyte sedimentation rate; **HAQ-DI** Health Assessment Questionnaire-Disability Index; **VAS** Visual Analogue Scale.

Correlation between the change in biomarkers levels from baseline to week 8 and disease activity (DAS28) at week 8, 16 and 24 was assessed with Spearman's correlation (ρ) (Table 3). The normality of data distribution was assessed by Shapiro-Wilk test and significance between groups was assessed by Mann-Whitney nonparametric test. Data were presented as a mean with either 95% confidence interval (CI-95%), standard error of mean (SEM) or standard deviation of mean (SD). Statistical significance in mean biomarker change between treatment groups at baseline and week 8 were assessed by unpaired ANOVA t-test. Significance was considered when p-values were $p < 0.05$; *, $P < 0.01$; **, $p < 0.001$; ***, $p < 0.0001$; ****. All statistical analyses were performed by using MedCalc version 14.8.1 and Prism Graphpad version 8.1.2 for graphical preparation.

3. Results

3.1 Baseline Demographics

Table 2. Comparison of AMBITION cohort with Healthy controls in patient demographics and baseline biomarker levels. Differences between cohorts are shown as ANCOVA values adjusted for race differences.

Variables	AMBITION cohort RA Patients (N = 387)	Healthy Controls (N = 72)	Difference between cohorts
Age (years), mean (SD)	50.9 (12.9)	49.7 (6.1)	ns
Female, n (%)	300 (76)	41 (76)	ns
Race, no. white/black/other (%)	325 (84)/14 (4)/48 (12)	51 (71)/21 (29) /0 (0)	<0.0001
Baseline C1M (ng/mL), mean (SD)	43.4 (30.7)	18.7 (15.3)	<0.001
Baseline C2M (ng/mL), mean (SD)	0.38 (0.2)	0.3 (0.16)	<0.001
Baseline C3M (ng/mL), mean (SD)	17.8 (8.0)	11.1 (2.8)	<0.001
Baseline C4M (ng/mL), mean (SD)	50.9 (24.9)	21.5 (6.1)	<0.001
Baseline CRPM (ng/mL), mean (SD)	20.9 (12.9)	7.5 (2.1)	<0.001
Baseline CRP (ng/mL), mean (SD)	3.0 (3.5)	-	

This current sub-study of AMBITION had 387 patients divided into three treatment arms with similar distribution of age, BMI, gender and disease activity (Table 1). There were no significant differences in baseline parameters between treatment groups except the number of swollen joint count (SJC) that was slightly higher in placebo patients ($P=0.04$) and lower baseline C4M levels in placebo patients ($P=0.02$). The same differences regarding clinical parameters were found in the complete AMBITION study (data not shown). All biomarkers except C2M correlated significantly at baseline ($P<0.0001$ to $P<0.01$, Spearman's rho 0.15 to 0.31) to the initial DAS28. The baseline levels of biomarkers in sub-study of AMBITION cohort were compared with healthy controls, involving 72 age and gender matched donors (Table 2). Comparison of these values was performed with ANCOVA adjusted for race differences and revealed a significantly lower baseline levels of all biomarkers in healthy controls ($P<0.001$).

3.2 Biomarker modulation at week 8

The week 8 levels of biomarkers associated with connective tissue remodeling, C1M and C3M, were significantly downregulated by Tocilizumab (TCZ) when compared to placebo ($P<0.05$ and $P<0.0001$, respectively) and C3M also by Methotrexate (MTX) compared to placebo ($P<0.01$) (Fig. 2A and 2C). The inhibition of C1M and C3M with TCZ was respectively 23% and 16% greater than that of MTX ($P<0.01$ and $P<0.0001$). The levels of the basement membrane remodeling biomarker C4M, were likewise inhibited by both TCZ and MTX comparing to placebo ($P<0.0001$ and $P<0.05$, respectively) and the effect of TCZ was 22% greater than MTX ($P<0.0001$) (Fig. 2D). In contrast, TCZ had minimal and MTX no effect on downregulation of the cartilage degradation biomarker C2M when compared to placebo (Fig. 2B). Considering effect of MTX on inflammatory biomarkers modulation, both CRP and CRPM levels were downregulated at week 8 when compared to placebo, 27% and 10% decrease respectively (Fig. 2E and 2F). TCZ shown greater than MTX inhibitory effect on CRP and CRPM resulting in 97% ($P<0.0001$) and 36% ($P<0.01$) decrease comparing to placebo patients.

3.3 Treatment dependent difference in biomarkers profiles of ACR50 responders and non-responders

Patients of the TCZ and MTX groups who achieved ACR50 at week 8 were classified as treatment responders. Patients that did not meet ACR50 at week 8 were classified as treatment non-responders. The treatment with TCZ demonstrated to be better than MTX with a higher ACR50 response rate ($P=0.01$). Among the 164 patients that were treated with TCZ, 46 patients reached ACR50 at week 8 (28%). In the MTX group, only 18 of the 161 patients reached ACR50 at week 8 (11%).

C1M among responders and non-responders in TCZ group did not show significant difference in the biomarker profiles at week 8 and 24, whereas MTX patients showed significant ($P<0.05$) difference in C1M levels at week 8 (23% decrease vs 2% increase) (Fig. 3A). There was a clear difference in profiles of C1M MTX patients once correlated to DAS28 at week 24, albeit statistically insignificant. Treatment with either TCZ or MTX did not result in significant differences in C2M profiles of responding and non-responding patients at week 8 and 24 (ns) (Fig. 3B). MTX showed a trend to increase the C2M level, whereas the level was unchanged with TCZ. C3M profiles did not display any significant differences after treatment with TCZ between responders and non-responders at week 8 and 24, however MTX patients

showed significant difference ($P<0.01$) in the decrease of C3M levels at week 8 (18% in responders vs 9% in non-responders) (Fig. 3C). The statistical significance between MTX C3M profiles increased ($P<0.001$) once correlated with DAS28 at week 24 (19% decrease in responders vs 4% in non-responders). Similarly, treatment with TCZ did not result in significantly different C4M profiles in responders and non-responders at both time points, while MTX patients displayed significant difference ($P<0.01$) in the decrease of C4M levels at week 8 (23% in responders and 9% in non-responders) with increased significance ($P<0.001$) once correlated with DAS28 at week 24 (21% in responders and 4% in non-responders) (Fig. 3D).

Treatment with TCZ did not result in significantly different CRPM profiles among responders and non-responders at week 8 and 24, but MTX patients displayed significantly different ($P<0.05$) CRPM profiles at week 8 (14% decrease in responders vs 2% increase in non-responders) (Fig. 4A). Intriguingly, the opposite profiles of CRPM was observed once correlated with DAS28 at week 24, 7% increase in responders and 3% decrease in non-responders in CRPM levels ($P<0.05$).

There was observed a strong modulation of the CRP levels among both treatment groups. Patients treated with TCZ displayed a significantly different ($P<0.05$) decrease of CRP profiles between responders and non-responders at week 8 (91% vs 59%) and after correlation to DAS28 at week 24 (76% vs 54%) (Fig. 4B). Similarly, treatment with MTX resulted in significantly different ($P<0.01$) CRP profiles between responders and non-responders at week 8 (56% decrease vs 9% increase) with increased significance ($P<0.001$) after correlation to DAS28 at week 24 (39% decrease vs 26% increase).

3.4 Correlation between change in biomarkers levels and change in disease activity at week 8, 16 and 24

To investigate whether differences in tissue degradation and tissue specific inflammation levels can be associated with overall improvement in disease activity, the difference in biomarkers levels were correlated to the change in disease activity score (DAS28) at week 8, 16 and 24. The prognostic value of measured biomarkers was assessed by Spearman's rank correlation coefficient (ρ) and p-value between change in tissue remodeling biomarkers from baseline to week 8 and change in disease activity (DAS28) at week 16 and 24 (Table 3). As a validation step, biomarker levels at baseline in the TCZ group were correlated to DAS28 change at week 16. These findings were validated by investigating correlation between week 8 biomarker levels with DAS28 change at week 24 in the PBO group. None of the investigated biomarker correlated to DAS28 in either TCZ or PBO group and no significant differences in correlation between both groups were observed (data not shown). In patients treated with Tocilizumab (TCZ), only CRP was significantly correlated with change in DAS28 at week 8 ($\rho=0.32$, $P<0.0001$), week 16 ($\rho=0.31$, $P=0.0001$) and week 24 ($\rho=0.21$, $P=0.01$) (Table 3). In patients treated with Methotrexate (MTX), four biomarkers correlated with change in DAS28 at all time points (C3M, C4M, CRP and CRPM). Change in C3M levels correlated to change in DAS28 at week 8 with correlation coefficient of 0.32 ($P=0.0001$) at week 8, 0.3 ($P=0.0005$) at week 16 and 0.26 ($P=0.003$) at week 24. Change in levels of C4M correlated with change in DAS28 with correlation coefficient of 0.35 ($P<0.0001$) at week 8, 0.32 ($P=0.0001$) at week 16 and 0.26 ($P=0.002$) at week 24. Change in levels of CRP correlated with change in DAS28 with correlation coefficient of 0.41 ($P<0.0001$) at week 8, 0.34 ($P<0.0001$) at week 16 and 0.25

(P=0.0026) at week 24. Moreover, change in levels of CRPM correlated significantly with change in DAS28 with correlation coefficient of 0.29 (P=0.0006) at week 8, 0.18 (P=0.039) at week 16 and 0.21 (P=0.016) at week 24.

Table 3. Correlation between week 8 change in levels of respective biomarkers according to the treatment group and change in disease activity (Δ DAS28) at week 8, 16 and 24. Correlation values are given as Spearman's correlation coefficient (ρ)

Biomarker	Tocilizumab		Methotrexate			
	Δ DAS28	Δ DAS28	Δ DAS28	Δ DAS28	Δ DAS28	Δ DAS28
	week 8	week 16	week 24	week 8	week 16	week 24
C1M	$\rho = 0.08$ P = 0.43	$\rho = 0.06$ P = 0.53	$\rho = 0.002$ P = 0.98	$\rho = 0.11$ P = 0.22	$\rho = 0.06$ P = 0.53	$\rho = 0.08$ P = 0.4
C2M	$\rho = 0.02$ P = 0.87	$\rho = 0.03$ P = 0.73	$\rho = 0.02$ P = 0.83	$\rho = 0.04$ P = 0.66	$\rho = -0.04$ P = 0.69	$\rho = 0.02$ P = 0.87
C3M	$\rho = 0.1$ P = 0.24	$\rho = 0.12$ P = 0.16	$\rho = 0.05$ P = 0.54	$\rho = 0.32$ P = 0.0001	$\rho = 0.3$ P = 0.0005	$\rho = 0.26$ P = 0.003
C4M	$\rho = 0.08$ P = 0.38	$\rho = 0.08$ P = 0.37	$\rho = -0.01$ P = 0.89	$\rho = 0.35$ P < 0.0001	$\rho = 0.32$ P = 0.0001	$\rho = 0.26$ P = 0.002
CRP	$\rho = 0.32$ P < 0.0001	$\rho = 0.31$ P = 0.0001	$\rho = 0.21$ P = 0.01	$\rho = 0.41$ P < 0.0001	$\rho = 0.34$ P < 0.0001	$\rho = 0.25$ P = 0.0026
CRPM	$\rho = 0.13$ P = 0.13	$\rho = 0.13$ P = 0.14	$\rho = 0.1$ P = 0.24	$\rho = 0.29$ P = 0.0006	$\rho = 0.18$ P = 0.039	$\rho = 0.21$ P = 0.016

4. Discussion

In this sub-study of the AMBITION trial we investigated association of different tissue remodeling biomarkers with aspects of joint degradation and inflammation in RA. We investigated the biomarker profiles in response to TCZ, MTX and placebo. In the full AMBITION study, clinical signs and symptoms after treatment with TCZ and MTX have been examined, but tissue remodeling biomarkers have not been investigated [27]. The approach involving tissue remodeling biomarker assessment gives a unique insight into the drug's mode of action at the tissue level and ongoing tissue remodeling processes.

Results of this study confirmed that investigated biomarkers are significantly upregulated in Rheumatoid Arthritis (Table 2), suggesting that an ongoing excessive ECM remodelling results in the excessive release of these biomarkers from the affected joint structures to circulation.

We demonstrated that there was significant difference between the two treatments and placebo patients in modulation of tissue remodeling biomarkers. Levels of C1M, C3M and C4M were significantly suppressed by TCZ compared to MTX, suggesting that TCZ exhibit more tissue protective effect towards different joint structures than that of MTX. TCZ had a minimal and MTX no effect on cartilage degradation measured by C2M. We observed a great inhibition of CRP and significant inhibition of CRPM after treatment with TCZ, whereas MTX had limited effect on their levels. This can be explained by the fact that IL-6 receptor is a main target for TCZ treatment and CRP is produced in the liver in the response to high levels of IL-6. Thus, TCZ should suppress CRP, which is observed and further downstream suppression of CRPM, which is observed as expected. The superior effect of TCZ in controlling of CRP and CRPM levels in the present study can be compared with LITHE study where TCZ inhibited completely the level of CRP and in the large extent level of CRPM [20]. These results indicate that TCZ is more effective than MTX treatment, that better limits tissue turnover and inflammation. More importantly, it indicates that measured biomarkers can be successfully applied to reveal differences in the mode of action between the investigated treatments.

We examined biomarker profiles of ACR50 responding and non-responding patients to investigate if any of the biomarker could stratify patients that benefited from treatments already at week 8. The significant differences in tissue remodeling biomarker profiles between treatment responders and non-responders were observed primarily among patients treated with MTX. This was an interesting observation considering that MTX patients had less biomarker downmodulation than TCZ patients. Despite the fact that TCZ patients were more likely to reach ACR50 than MTX patients, the significant differences in biomarker profiles discriminating responders from non-responders were observable only for CRP. Previously, similar studies have shown that combination treatment involving TCZ+MTX do not necessarily display differences in profiles of tissue turnover biomarkers between ACR50 responders and non-responders already at week 8 [20]. These differences were observable at week 16 and 24, which could explain results seen in TCZ patients. Interestingly, results of this study showed that separation of these treatments, allowed to observe differences in biomarker profiles of patients treated with MTX monotherapy as early as at week 8. This indicates that the neo-epitope biomarkers can be potentially used as a tool to identify treatment responding and non-responding patients at the early treatment time point.

Biomarkers of tissue turnover have shown a great prognostic potential and can predict long-term clinical response to treatment already at the early time-point [26]. It is of great importance to determine at the possibly earliest stage whether patient will respond to the treatment in the future and benefit from the treatment. We demonstrated that only change to week 8 in the CRP levels significantly predicted clinical response to TCZ and correlated with the change in the disease activity at week 16 and 24 as a response to treatment (Table 3). Interestingly, more biomarkers were predictive for MTX treatment. In this case, change to week 8 in the C3M, C4M, CRP and CRPM levels significantly predicted clinical response at week 16 and 24.

These results show a great value of the biomarkers which can be a determining indicator of patient clinical benefit from the given treatment. Previous studies have shown that biomarker levels reflecting structural joint damage such as C1M, C2M and C3M can be associated with disease progression and disease activity over time [22][32].

Our results confirm similar biomarker analysis performed for LITHE study that measured C2M, C3M, CRPM and CRP, but in patients treated with combination therapy involving TCZ at two doses (8 and 4 mg/kg) with MTX. The combination therapy of TCZ and MTX decreased C3M and CRPM levels at week 8 in a comparable extent to TCZ alone in our study [20]. There was a greater decrease in levels of C2M and CRP at week 8 after TCZ+MTX treatment, albeit TCZ monotherapy significantly decreased levels of CRP proving its positive effect on inflammation process. We observed a limited effect of TCZ alone on cartilage balance measured with C2M, however high C2M upregulation is typical at erosive disease stages and that was not a case in the examined cohort. Another study involving the LITHE cohort, investigated the effect of structural joint damage and progression, measured with C1M [22]. TCZ in combination with MTX decreased levels of C1M at week 8 in a greater extent than TCZ monotherapy, but both treatments provided significant downmodulation of C1M and protective effect on connective tissues. Gudmann et al. investigated the baseline membrane remodeling measured with C4M after TCZ+MTX treatment in LITHE and RADIATE cohorts [23]. Reported results support our findings, where TCZ+MTX treatment downregulated levels of C4M at week 8 in the similar extent as TCZ monotherapy, proving that both treatment strategies have protective effect on tissue remodeling. These results indicate that TCZ monotherapy can be successfully applied as an effective alternative to commonly used combined therapy involving TCZ+MTX. One of the main limitations of this study was that we did not measure biomarkers at other time points than baseline and week 8, so we could not anticipate the long-term treatment effect on biomarker modulation. Another limitation of this study was no radiographics to support our findings of tissue remodeling. However, radiographics showed sensitivity limitations in detecting early structural changes in joints and surrounding structures, indicating their limited applicability in this type of study [33].

5. Conclusion

Patients receiving TCZ treatment for 8 weeks have clearly higher suppression of tissue remodeling and inflammatory biomarkers over patients treated with MTX. The decrease of the inflammatory process is believed to be associated with decrease of ECM remodeling and tissue degradation, which is supported by this study. On the other hand, patients receiving MTX enable for a clear discrimination of biomarker profiles and identification of those who were most likely to benefit from the treatment at the early time point. The results of this study highlight that biomarkers reflecting tissue turnover can be applied as disease and response to therapy markers. Their application may facilitate future identification of patients most likely to benefit from a given treatment with the aim to develop possibly best personalised treatment strategy.

Abbreviations

ACR50 - 50% Disease Improvement

BMI - Body Mass Index

C1M - Type I Collagen Degradation Biomarker

C2M - Type II Collagen Degradation Biomarker

C3M - Type III Collagen Degradation Biomarker

C4M - Type IV Collagen Degradation Biomarker

CRP - C-Reactive Protein

CRPM - C-Reactive Protein Degradation Biomarker

CV - coefficient of variance

DAS28 - Disease Activity Score in 28 joints

DMARDs - Disease-Modifying Anti-Rheumatic Drugs

ECM - Extracellular Matrix

IL-6 - Interleukin-6

LLOD - Lower Limit of Detection

MMP - Matrix Metalloproteinase

MTX - Methotrexate

PBO – Placebo

RA - Rheumatoid Arthritis

TCZ - Tocilizumab

TMB - 3,3',5,5' - tetramethylbenzidine substrate

ULOD - Upper Limit of Detection

Declarations

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The AMBITION study (NCT00109408) was approved by the ethics committee at each participating institution (in USA, Canada and Israel) and was conducted in accordance with the Principles of Good Clinical Practice and according to the Declaration of Helsinki. All patients included provided written, informed consent before inclusion in the study.

CONSENT FOR PUBLICATION

Not Applicable.

AVAILABILITY OF DATA AND MATERIALS

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

COMPETING INTERESTS

Anne C. Bay-Jensen (ACBJ), Morten Karsdal (MK), Anne Siebuhr (ASO) and Samra Sardar (SSA) are full-time employees and/or shareholders at Nordic Bioscience. Patryk Drobinski (PAD) is a full-time employee at University of Copenhagen. PAD have no disclosures and no competing interests. None of the authors received any kind of financial benefits or other bonuses for the work described in this manuscript.

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This work was supported by the University of Copenhagen (KU) and Nordic Bioscience. The KU was not involved in the study design, collection, analysis and interpretation of data, writing or deciding to submit the manuscript for publication. Nordic Bioscience was involved in the study design, collection, analysis, interpretation of data, writing the manuscript and the decision to submit the manuscript for publication.

AUTHORS' CONTRIBUTIONS

All authors were involved with the design of the study, interpretation of data, critical revising of the manuscript and approving the final version for submission. ACBJ, ASO and MK were primarily responsible for the data acquisition, whereas PAD and SSA primarily did the analysis of the data and drafted the manuscript. PAD (pad@nordicbio.com) and ASO (aso@nordicbio.com) take full responsibility of the integrity of the work from inception to finished article.

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Figures

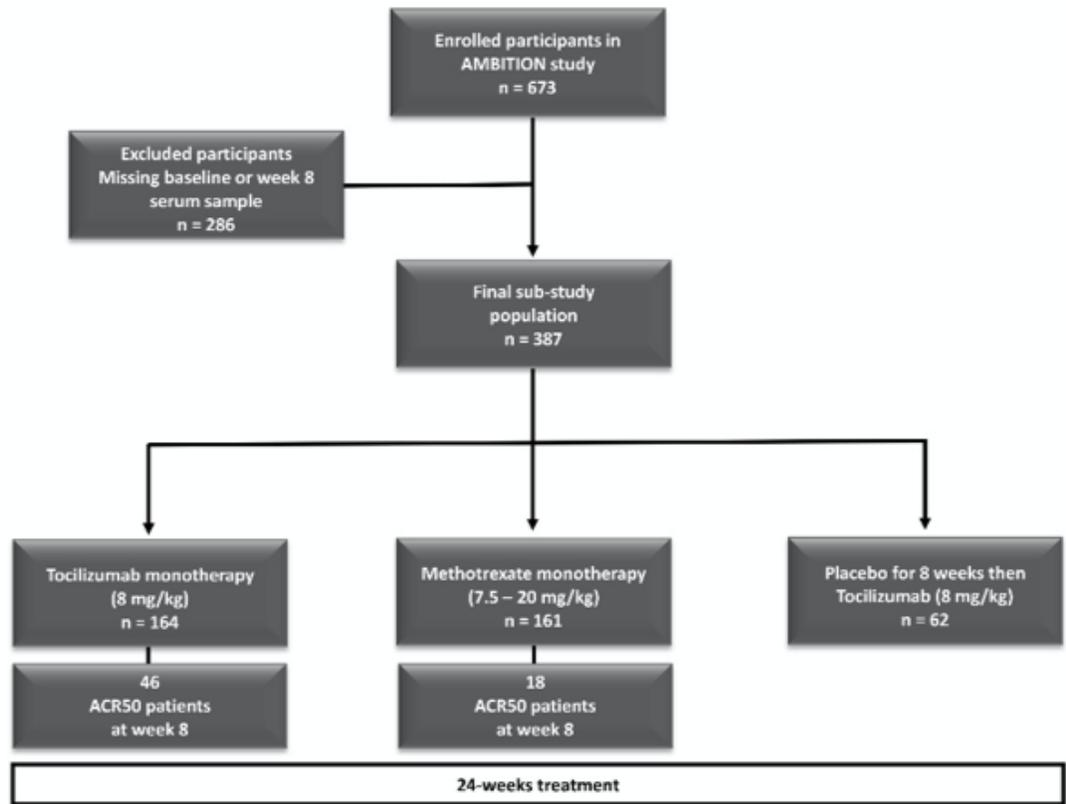


Figure 1

Patients randomisation into three different treatment arms in the AMBITION study.

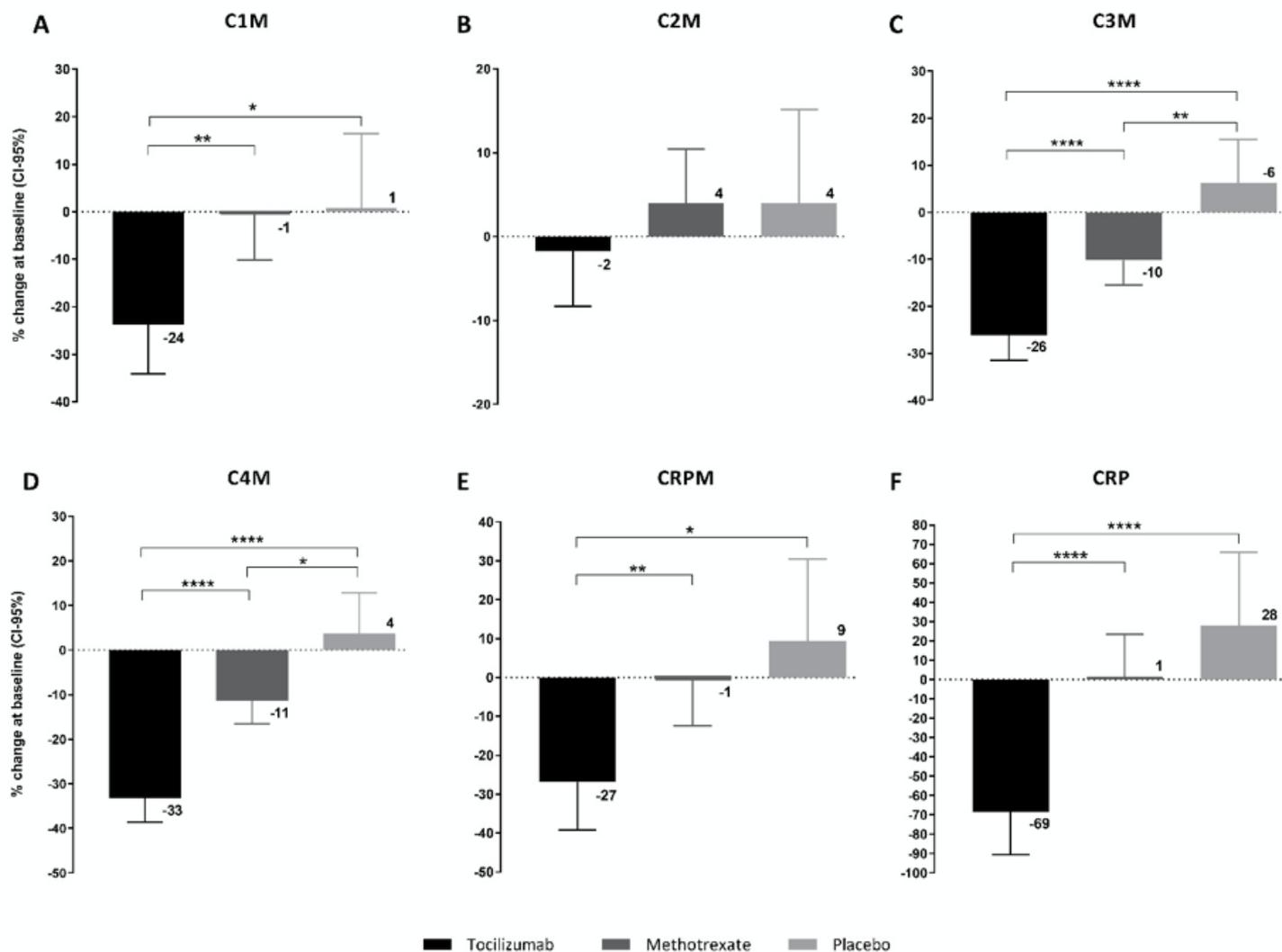


Figure 2

Figures represent differences in modulation of tissue remodelling biomarkers C1M (A) (TCZ n = 115; MTX n = 128; PBO n = 49), C2M (B) (TCZ n = 109; MTX n = 122; PBO n = 36), C3M (C) (TCZ n = 141; MTX n = 145; PBO n = 47), C4M (D) (TCZ n = 140; MTX n = 146; PBO n = 46), CRPM (E) (TCZ n = 143; MTX n = 145; PBO n = 48) and CRP (F) (TCZ n = 164; MTX n = 161; PBO n = 53) according to treatment from baseline to week 8 (%). Data are shown as mean with confidence interval CI-95%.

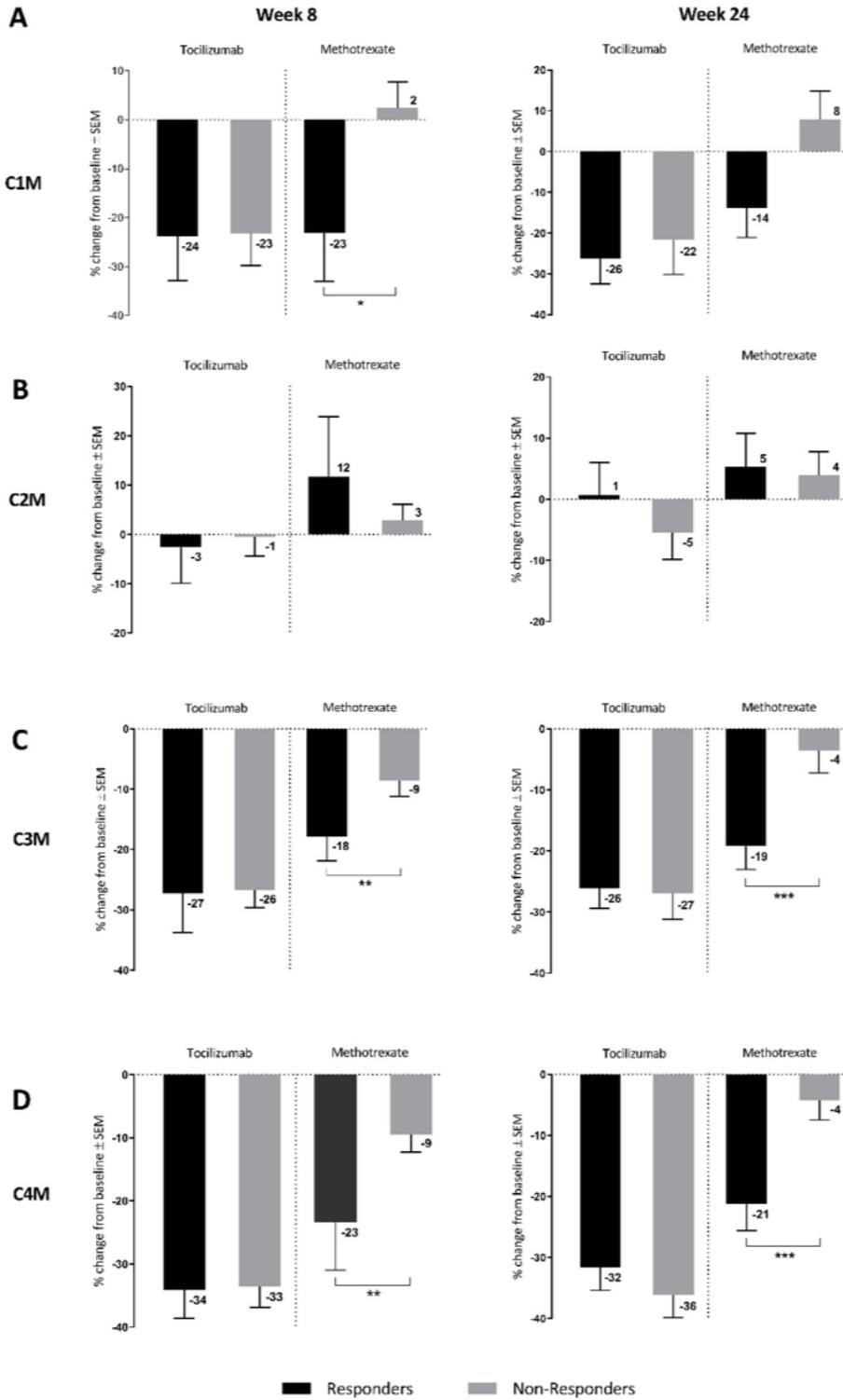


Figure 3

Treatment dependent difference in degradation biomarker profiles of patients assigned as ACR50 responders (black bars) and ACR50 non-responders (grey bars) from baseline to week 8 and predictive profiles at week 24. Figures represent serum levels of C1M (A) (TCZ n = 115; MTX n = 128), C2M (B) (TCZ n = 109; MTX n = 122), C3M (C) (TCZ n = 141; MTX n = 145), C4M (D) (TCZ n = 140; MTX n = 146) in

response to TCZ and MTX at week 8 and predictive profiles at week 24. Data are shown as mean with SEMs.

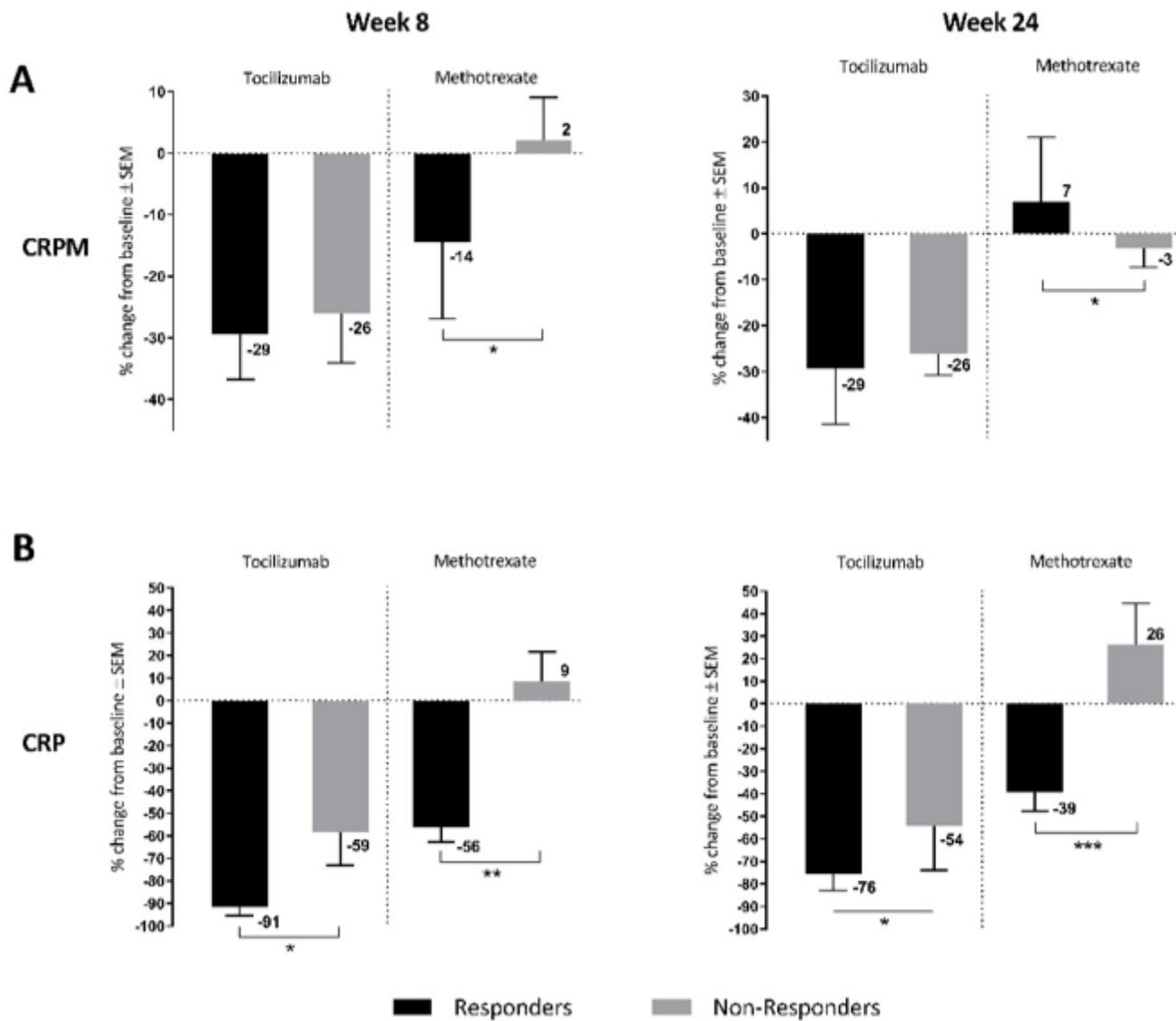


Figure 4

Treatment dependent difference in inflammatory biomarker profiles of patients assigned as ACR50 responders (black bars) and ACR50 non-responders (grey bars) from baseline to week 8 and predictive profiles at week 24. Figures represent serum levels of CRPM (A) (TCZ n = 143; MTX n = 145), CRP (B) (TCZ n = 164; MTX n = 161) in response to TCZ and MTX at week 8 and predictive profiles at week 24. Data are shown as mean with SEMs.