# Type IV collagen metabolism is associated with disease activity, radiographic progression and response to tocilizumab in rheumatoid arthritis

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## Abstract

### Objective

*The expanding spectrum of targeted therapies for rheumatoid arthritis (RA) implies a need for development of precision tools for disease assessment reflecting pathobiologic processes. Type IV collagen is an abundant protein of basement membranes, but is also present in the intercellular matrix of the synovial lining layer. We aimed to investigate the association of type IV collagen turnover with RA disease activity, response to IL-6 inhibition and radiographic progression.*

### Methods

*C4M, a serologic marker of type IV collagen metabolism, was measured at baseline and at follow-up in serum samples of RA patients participating in the phase III studies LITHE (n=687) and RADIATE (n=217). Both were double-blinded, placebo-controlled clinical trials testing the safety and efficacy of 4 and 8 mg/kg tocilizumab (TCZ) in combination with methotrexate (MTX) vs. MTX plus placebo. Associations with disease activity, radiographic severity and ACR response were investigated.*

### Results

*Baseline C4M correlated significantly with clinical disease parameters in both study populations, including DAS28, HAQ score and VASpain (all $p<0.00001$). C4M at baseline correlated significantly with change in JSN ($p=0.001$) and Sharp score ($p=0.00002$) at 52 weeks. TCZ lowered C4M by 11–40% in a dose dependent manner. The likelihood of achieving an ACR20 response by week 16 was associated with C4M suppression exceeding the median decrease at week 4 ($p<0.0001$).*

### Conclusion

*Type IV collagen remodelling was associated with disease activity and radiographic progression in RA and was persistently and dose-dependently suppressed by TCZ. These findings indicate that C4M may serve as a plausible biologic marker of destructive synovitis growth in RA.*

**Key words**
rheumatoid arthritis, biomarkers, type IV collagen, basement membrane

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*Received on October 2, 2017; accepted in revised form on January 31, 2018.*



*Funding: this study was supported by research grants from the region of Southern Denmark and Department of Rheumatology C, Odense University Hospital, Denmark. The research leading to these results has received partial funding from the European Union Seventh Framework Programme (FP7/2007–2013) under grant agreement no. 305815 and partial funding from the Danish Research Foundation (DFF under act 2013).*

*Competing interests: N.S. Gudmann, P. Juhl, C. Thudium, A.S. Siebuhr, I. Byrjalsen, A.C. Bay-Jensen and M.A. Karsdal are full-time employees at Nordic Bioscience. M.A. Karsdal and A.C. Bay-Jensen hold stock in Nordic Bioscience. Nordic Bioscience is a privately owned, small medium-sized 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. P. Junker has nothing to disclose. The biomarker part of the study was supported by the Danish Research Foundation.*

## Introduction

Rheumatoid arthritis (RA) is an autoimmune, systemic disease, which is primarily manifested in peripheral joints leading to deformities and loss of function if undertreated (1)(2). The currently prevailing "treat-to-target" strategy aiming at prompt synovitis suppression at all disease stages using synthetic or biologic disease-modifying anti-rheumatic drugs (DMARDs) has significantly improved the prognosis for RA patients regarding joint preservation and longevity (3). However, the clinical RA disease profile is quite variable and up to 30% of patients do not respond adequately to the DMARD of first choice. This leads to delayed disease control for some patients, while others may be over treated (4, 5). Thus, there is a need to develop new tools to understand and monitor key disease processes in order to enable clinicians to offer personalised treatment strategies based on pathobiologic insight.

Extracellular matrix (ECM) proteins of joints and adjacent structures are targets as well as adaptive defense mediators. Previous studies have shown that altered remodelling of the synovial membrane and bone is reflected in the serum or synovial fluid levels of type I and III collagen degradation metabolites (6). The synovial membrane does not contain a basement membrane; however, it does contain basement membrane components including type IV collagen within the interstitial matrix of the lining layer (7).

Type IV collagen turnover can be assessed in serum by measuring specific soluble metabolites generated by matrix metalloproteinase cleavage at amino acid 162 located in the 7S associated domain of α1 (IV) (8). Increased serum levels of C4M have previously been reported in liver fibrosis (9), lung fibrosis (8) (10) and colorectal cancer (11).

Tocilizumab (TCZ) is a humanised anti-IL-6 receptor monoclonal antibody, which significantly reduces swollen and tender joint counts, as well as radiographic progression in active RA (12). We hypothesised that type IV collagen turnover is increased in active RA, associates with disease activity measures and can be modulated by TCZ. The aims were to study type IV collagen turnover as assessed by C4M in serum samples of the LITHE (ClinicalTrials.gov identifier: NCT00106535) and RADIATE (ClinicalTrials.gov identifier: NCT00106522) studies. Specifically, we investigated the associations with disease activity measures, radiographic progression and treatment response to TCZ.

## Material and methods

*Study design and serum samples*

The present work is a biomarker sub study of LITHE (n=687) comprising serum samples from a 1-year, double-blinded treatment study, where 1149 patients were randomised 1:1:1 and assigned to one of three treatment groups: 4-mg/kg TCZ (TCZ4), 8-mg/kg TCZ (TCZ8) or placebo (PBO) in combination with a stable dosage of MTX (10–25mg/week) (12). TCZ and PBO were administered intravenously every 4 weeks. Patients who experienced <20% improvement from baseline in the swollen joint counts (SJC) and tender joint counts (TJC) at week 16 or later, could receive blinded rescue therapy in a stepwise fashion between week 16 and 28. For patients receiving rescue therapy: The first-step of blinded rescue, patients receiving PBO + MTX switched to TCZ 4mg/kg + MTX, patients receiving TCZ 8mg/kg + MTX remained on TCZ 8mg/kg + MTX. Second-step rescue consisted of TCZ 8mg/kg + MTX for all patients, regardless of initial treatment, and was offered through week 52. Patients who had an inadequate response after three doses of the first-step rescue discontinued treatment. According to the study protocol, serum for biomarker research was collected from patients who provided written informed consent. Patients with a baseline serum sample, and at least one sample from 4, 16, 24 or 52 weeks were included.

The RADIATE study (n=217) (clinical Trial.gov identifier: NCT00106522. Registered March 25, 2005) has previously been reported by Emery *et al.* (13). In short, it is a randomised, double-blinded, placebo-controlled, parallel-group, phase III study. Patients had moderate to severely active RA with inadequate response to one or

**Table I.** Demographics at baseline of the 687 LITHE and 217 subjects of RADIATE.

| | LITHE | | | | RADIATE | | | |
|---|---|---|---|---|---|---|---|---|
| | PBO (n=238) | TCZ 4mg (n=228) | TCZ 8mg (n=221) | All (n=687) | PBO (n=65) | TCZ 4mg (n=72) | TCZ 8mg (n=80) | All (n=217) |
| Age, years | 51.8 (50.2-53.4) | 51.6 (49.9-53.3) | 54.4 (52.9-55.9) | 52.6 (51.7-53.5) | 53.6 (51.5-55.7) | 51.6 (48.7-54.5) | 53.1 (50.6-55.6) | 52.9 (51.29-54.4) |
| Gender, Female % | 83% | 86% | 79% | | 75% | 82% | 88% | 82.1% |
| BMI | 28.2 (27.3-29.2) | 27.4 (26.6-28.2) | 27.4 (26.7-28.1) | 27.7 (27.3-28.2) | 27.6 (26.6-28.6) | 28.5 (27.1-29.9) | 26.8 (25.4-28.2) | 27.8 (26.9-28.7) |
| Disease duration, mean years (95%-CI) | 9.2 (8.1-10.2) | 10.2 (9.1-11.2) | 9.6 (8.5-10.8) | 9.6 (9.0-10.3) | 11.3 (9.9-12.8) | 11.7 (9.4 -14.0) | 13.4 (11.3-15.5) | 12.4 (11.1-13.7) |
| CRP, mg/dl | 2.2 (1.8-2.5) | 1.9 (1.6-2.2) | 2.2 (1.8-2.5) | 2.1 (1.9-2.3) | 3.8 (3.2-4.4) | 3.7 (2.8-4.7) | 3.2 (2.4-4.0) | 3.6 (3.1-4.1) |
| HAQ | 1.5 (1.5-1.6) | 1.5 (1.4-1.5) | 1.5 (1.4-1.6) | 1.5 (1.5-1.6) | 1.7 (1.6-1.8) | 1.7 (1.6-1.8) | 1.7 (1.6-1.8) | 1.7 (1.6-1.8) |
| VAS pain | 55.1 (52.3-57.8) | 52.8 (49.9-55.7) | 55.8 (52.7-58.8) | 54.5 (52.9-56.2) | 64.2 (60.8-67.7) | 63.5 (60.1-67.0) | 65.3 (60.8-69.8) | 64.4 (61.7-67.1) |
| DAS28 | 6.5 (6.4-6.7) | 6.5 (6.4-6.6) | 6.5 (6.4-6.7) | 6.5 (6.4-6.6) | 6.8 (6.6-7.0) | 6.8 (6.6 -6.9) | 6.8 (6.5-7.0) | 6.8 (6.7-6.9) |
| C4M | 99.5 (93.6-105.4) | 93.8 (89.0-98.7) | 98.6 (91.8-105.5) | 97.3 (93.9-100.7) | 79.0 (66.5-90.2) | 73.3 (69.4-82.6) | 75.6 (66.1-87.5) | 75.9 (66.6-88.2) |

Values are shown as arithmetic mean with 95% confidence interval (CI) in parentheses.

more TNF antagonists within the past year. Patients were randomised 1:1:1 to 4-mg/kg TCZ, 8-mg/kg TCZ or PBO in combination with a stable dose of MTX (10-25mg/week) for 24 weeks. Stable oral corticosteroids (10 mg/d prednisone or equivalent) or non-steroidal anti-inflammatory drugs were permitted throughout the study. For these exploratory analyses patients with a baseline sample and a sample from week 16 were included implying that samples of 217/499 patients were analysed.

Serum samples of both studies were stored at temperatures below -70°C until assayed. The studies were approved by the ethics committee at each participating institution and all patients provided written informed consent (12, 13). The studies were conducted according to the Principles of Good Clinical Practice and according to the Declaration of Helsinki. Patients included in the biomarker sub study consented to the use of their samples for assessment of markers related to joint biology and inflammation.

*Measurement of the type IV collagen metabolite C4M*

Type IV collagen turnover was quantified in serum by means of the competitive ELISA assay C4M (8) (Nordic Bioscience, Herlev, Denmark). All samples were measured in duplicate and in a blinded manner. In brief, a 96-well streptavidin plate was coated with biotinylated synthetic peptide of the sequence biotin-ILGHVPGMLLK dissolved in PBS buffer (2mM $KH_2PO_4$, 9mM $Na_2HPO_4$, $2H_2O$, 3mM KCl, 137 mM NaCl, pH 7.4) and incubated for 30 minutes at 20°C by shaking at 300 rounds per minute (rpm). Twenty microliters of peptide calibrator (IL-GHVPGMLLK) or sample dissolved in assay buffer (25mM Tris, 1% BSA, 0.1% Tween 20, pH 7.4) were added to appropriate wells, followed by 100ul of the appropriate conjugated monoclonal antibody, and incubated for one hour at 20°C and shaking at 300rpm. Each incubation was followed by five times wash in washing buffer (20mM Tris, 50mM NaCl, pH 7.2). Finally, 100ul of tetramethylbenzidine (TMB) (cat. No. 438OH; Kem-En-Tec, Copenhagen, Denmark) was added, and plates were incubated for 15 minutes at 20°C in the dark while shaken at 300rpm. The TMB reaction was then stopped by adding 100ul of stopping solution (10mM $H_2SO_4$) and measured spectrophotometrically at 450nm with 650nm as reference. Different production batches were used for measurement of the LITHE and RADIATE samples, but quality control samples were included in the production of the kits to ensure low batch-to-batch variability.

*Statistics*

Summary statistics were used for general demographics, and disease characteristics at baseline. Differences between the two treatment groups TCZ4 and TCZ8 compared to placebo were assessed by one-way ANOVA using log-transformed data. The p-values were adjusted for multiple comparisons by the Dunnett's test. Spearman's ranked correlation test was conducted at baseline between serum C4M and age, body mass index (BMI), disease duration, visual analogue scale (VAS) pain, disease activity score (DAS28)-erythrocyte sedimentation rate (ESR), health assessment questionnaire (HAQ), joint space narrowing (JSN), Sharp score (SHP) and erosion score (ERN). Multiple regression analysis was performed on log-transformed data for structural progression (ΔJSN, ΔSHP and ΔERN score), with age, body mass index (BMI), disease duration, baseline CRP and baseline structural status as confounders. Changes from baseline C4M levels were studied as a function of time and treatment. Differences between the levels of C4M at each time point were compared by two-way ANOVA. Graphs depict the mean value (± SEM). All statistical analyses and Graphs were performed using SAS software v. 9.1.3, MedCalc Software v. 14.8.1 (MedCalc Software bvba, Ostend, Belgium) and Prism GraphPad v. 6.02.

## Results

*Demographics*

The LITHE and RADIATE biomarker sub-studies included 687 patients with an inadequate response to MTX therapy and 217 patients with an inadequate response to TNF inhibitors, respectively. Patients were equally distributed between the treatment arms (Table I). Mean age and BMI were similar across arms and for the two studies. The majority of patients were females. Mean dis-

ease duration was around 10 years and disease activity as assessed by DAS28 was without intergroup differences. CRP, HAQ, VAS pain and DAS28 were higher in RADIATE than in LITHE, probably reflecting different inclusion criteria. Radiographs were only scored in the LITHE study. The RA patients from both RADIATE and LITHE had higher C4M levels as compared to the level in healthy individuals of 63.2ng/ml (confidence interval 95%: 59.5–66.9ng/ml) reported by Kehlet *et al*. (11).

*Pretreatment type IV collagen turnover is associated with disease activity and structural joint damage*

Serum C4M levels were positively correlated with baseline DAS28 ($r_{LITHE}$=0.30 and $r_{RADIATE}$=0.32, $p$<0.0001), CRP ($r_{LITHE}$=0.64 and $r_{RADIATE}$=0.58, $p$<0.0001), HAQ ($r_{LITHE}$=0.24 and $r_{RADIATE}$=0.21, $p_{LITHE}$<0.0001 and $p_{RADIATE}$<0.0025, respectively) and VAS pain ($r_{LITHE}$=0.21 and $r_{RADIATE}$=0.30, $p$<0.0001) in both studies. In LITHE, serum C4M was significantly associated with JSN (r=0.13, $p$=0.001), ERN (r=0.14, $p$=0.0002) and SHP (r=0.14, $p$=0.0002) (Table II). By contrast, there were no correlations between serum C4M measured at baseline and age, gender or BMI in any of the studies.

*The association between basement membrane turnover and structural joint damage progression*

The association between basement membrane turnover at baseline and radiographic progression at 52 weeks was investigated in the PBO group from LITHE. Patients with a JSN, ERN or SHP score of 20 or more were excluded in the analysis as these were thought to be less likely to progress further. Baseline C4M was significantly associated with changes in JSN, ERN and SHP with correlation coefficients of 0.41, 0.54 and 0.59 with $p$<0.0001 (Table III). These correlations were only marginally affected after adjustment for BMI, age, disease duration, DAS28 and radiographic score at baseline.

*Dose-dependent inhibition of basement membrane turnover by tocilizumab*

TCZ8 significantly ($p$<0.0001) suppressed C4M serum levels by 30% from week 4 and onward when compared to the PBO group (Fig. 1A). In addition, TCZ reduced C4M by 15% compared to baseline from week 16 and on ($p$<0.001) (Fig. 1A). At week 16, C4M levels were significantly lowered by 38% and 22% of the baseline levels with TCZ8 and TCZ4 in LITHE (Fig. 1B). The same range of suppression was observed in RADIATE where TCZ8 and TCZ4 significantly lowered C4M by 24% and 11%, respectively (Fig. 1C).

*Association between change in basement turnover and early response to treatment*

Patients were categorised into two groups according to the median percentage C4M suppression at 4 or 16 weeks. In LITHE, patients in whom C4M levels were suppressed by more than median suppression at week 4 had an odds ratio of 2.1 ($p$<0.0001) for achieving an ACR20 response at week 16 compared to patients with a smaller treatment response (Fig. 2A). C4M suppression exceeding the median at week 16 was associated to an odds ratio of 2.1 for achieving an ACR20 response at week 16 compared to patients with a lower C4M decrease ($p$<0.0001) (Fig. 2B). In RADIATE the odds ratio for achieving an ACR20 response after 16 weeks was 2.6 ($p$=0.0004) for patients with the highest level of C4M suppression (Fig. 2C). Thus, early C4M suppression predicted a favourable clinical response to treatment.

**Table II.** Correlation between baseline C4M and clinical scores.

| | LITHE | | | RADIATE | | |
|---|---|---|---|---|---|---|
| | r | *p* | n. | r | *p* | n. |
| Age, years | ns | ns | 687 | ns | ns | 217 |
| Gender, Male % | ns | ns | 687 | ns | ns | 217 |
| BMI | ns | ns | 680 | ns | ns | 217 |
| Disease duration, mean years (95%-CI) | ns | ns | 686 | ns | ns | 217 |
| Baseline CRP, mg/dl | 0.64 | <0.0001 | 686 | 0.58 | <0.0001 | 217 |
| HAQ | 0.24 | <0.0001 | 623 | 0.30 | 0.0025 | 214 |
| VAS pain | 0.21 | <0.0001 | 679 | 0.32 | <0.0001 | 217 |
| DAS28 | 0.30 | <0.0001 | 672 | 0.32 | <0.0001 | 217 |

**Table III.** The correlation between type IV collagen turnover (C4M) at baseline and radiographic progression.

| PBO | $r_{partial}$ | *p* |
|---|---|---|
| Change JSN 52 weeks (n=79) | | |
| C4M alone | 0.41 | 0.0001 |
| C4M adjusted for BMI, Age, disease duration and DAS28 | 0.38 | 0.001 |
| C4M adjusted for BMI, Age, disease duration, DAS28 and JSN baseline | 0.35 | 0.004 |
| Change ERN 52 weeks (n=73) | | |
| C4M alone | 0.54 | <0.0001 |
| C4M adjusted for BMI, Age, disease duration and DAS28 | 0.53 | 0.0001 |
| C4M adjusted for BMI, Age, disease duration, DAS28 and SHP baseline | 0.51 | 0.0002 |
| Change SHP 52 weeks (n=52) | | |
| C4M alone | 0.59 | <0.0001 |
| C4M adjusted for BMI, Age, disease duration and DAS28 | 0.61 | <0.0001 |
| C4M adjusted for BMI, Age, disease duration, DAS28 and SHP baseline | 0.60 | <0.0001 |

A multiple regression model was applied on log transformed C4M baseline data, and data were adjusted according to age, BMI, sex and disease duration.

## Discussion

This study shows that C4M, a serologic marker of type IV collagen metabolism, correlates with disease activity, structural joint damage and radiographic progression in patients with active, long-standing RA. C4M decreased significantly in a time and dose dependent manner following IL-6 inhibition with tocilizumab. Patients with high baseline C4M levels and rapidly declining C4M following treatment initiation were more likely to achieve an ACR20

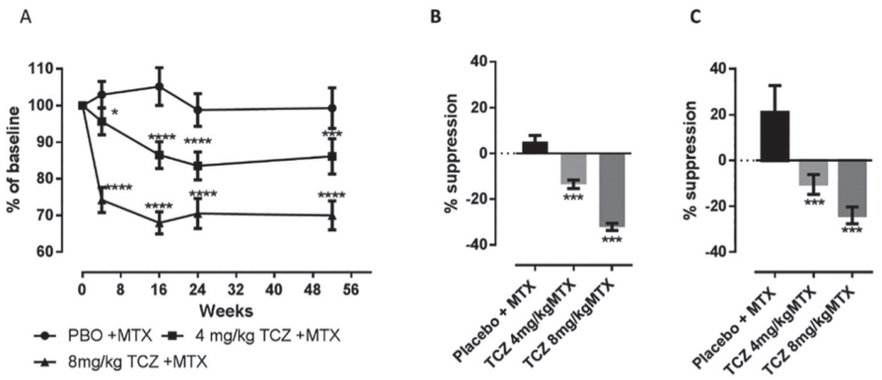


**Fig. 1.** Basement membrane remodelling measured by C4M was suppressed by TCZ.
**A**: Percentage change from baseline to week 4, 16, 24 and 54 for each of the 3 treatment groups in LITHE. **B**: Suppression of C4M week 16 in LITHE. **C**: Suppression of C4M week 16 in RADIATE. Error bars are shown as SEM. Significant levels are depicted **p*<0.05, ***p*<0.01, ****p*<0.001, *****p*<0.0001.

response at week 16 than those with a protracted decrease.

Type IV collagen is the most abundant non-fibrillar collagen in human tissues and is an important structural component of basement membranes where it exerts structural and barrier functions as well as cell regulatory, developmental and regenerative activities (14-16). Although the normal synovial membrane lacks a regular basement membrane, type IV collagen is present in the intercellular synovial lining matrix and in vascular basement membranes of the normal lining layer (7, 17). However, while the vascular basement membrane in the synovial lining layer contains all type IV collagen alpha-chains as assessed by immunohistochemical stainings, the alpha-chain profile is limited within the interstitial matrix of the synovial lining layer. Notably, in RA the type IV collagen alpha-chain composition was similar to controls in the synovial blood vessels, whereas the interstitial collagen type IV alpha-chain expression was markedly reduced or absent within the intercellular lining matrix (7). These authors also reported that the proportion of type A lining cells of the macrophage lineage capable of expressing collagenases including MMP-2 and 9 is increased at the cost of collagen type IV producing type B cells so that local degradative pathways may offset type IV collagen production (7). These findings indicate that depletion of type IV collagen in the intercellular matrix of the synovial lining layer is a major determinant for the serum level of C4M in RA patients.

In the RA joint, angiogenesis, *i.e.* formation of new capillaries within the preexisting microvasculature, is an early event which enhances leukocyte and other cell trafficking into the inflammatory infiltrate leading to synovial proliferation and joint destruction (18). Interleukin-6 (IL-6) together with IL-17 are important proangiogenic mediators in RA (19). Although the type IV collagen alpha-chain composition of the synovial vasculature was virtually normal in RA as assessed by chain specific immunolabelling (7), enhanced type IV collagen remodelling due to new blood vessel formation may also contribute to the C4M serum level in RA. Thus, the transition of type IV collagen fragments to the blood stream is facilitated by the dense microvascular network of the inflammatory infiltrate, particularly at the pannus-cartilage junction (20). This notion is further favored by the close correlations between baseline C4M and radiographic structural joint damage which is largely driven by pannus destructive growth (20). The parallel declines in C4M and x-ray progression during TCZ treatment, which has been reported to have angiostatic properties (21), supports this mechanistic concept. The association patterns of C4M with clinical and imaging findings before and during TCZ treatment clearly indicate that circulating C4M reflects core disease processes within the inflamed joints including those causing synovial swellings, structural damage and erosive progression. Although the origin of C4M in RA patients´ serum cannot be definitely settled from these associations, the synovial origin is

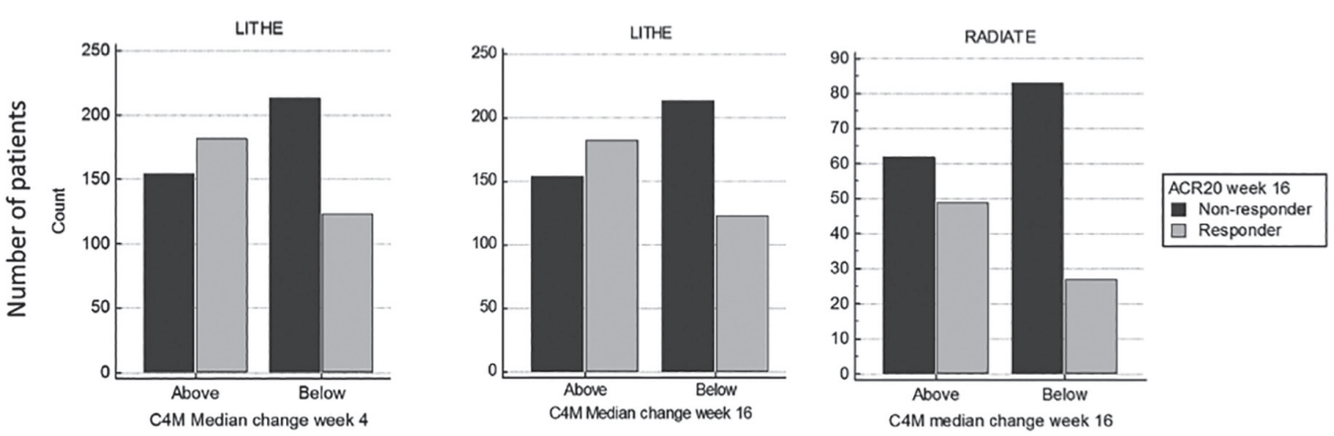


**Fig. 2.** Suppression of C4M was associated with ACR20. **A**: Frequency of ACR20 responders/non-responders (week 16) LITHE when separated according to C4M suppression at week 4 for above/below median. **B**: Frequency of responders in LITHE according to C4M suppression above/below median at week 16. **C**: Frequency of ACR20 responders/non-responders in RADIATE according to C4M suppression above/below median at week 16.

substantiated by data from a smaller study by Hørslev-Petersen *et al*. (22). These authors found, that the serum concentration of the 7S fragment of type IV collagen was increased in patients with active, long-standing RA. Moreover, they found that the synovial fluid:serum ratio of the 7S fragment was increased amounting to 2:1, which adds to the concept that the inflamed synovial membrane is a significant source of type IV collagen markers in the systemic circulation of RA patients with active disease (22).

Importantly, co-occurring pathologies involving type IV collagen containing tissues should also be considered as potential sources of type IV collagen seromarkers in RA. Thus, it has long been recognised that RA patients have increased cardiovascular morbidity and mortality compared to individuals without RA (21). Moreover, it has been reported that RA patients with active disease have significantly higher levels of cardiovascular disease (CVD) risk markers, intima-media thickness and arterial stiffness in particular, than patients in remission, indicating that there is a link between joint inflammation and systemic tissue responses (23). Of additional note, a treat-to-target strategy aiming at maximal synovitis suppression leads to improved cardiovascular health (24). Therefore altered type IV collagen remodelling in large vessels with incipient or ongoing arteriosclerosis should be considered as a possible further contribution to the C4M serum level in RA. To our knowledge only one study has addressed this issue. Thus, Ramazani *et al*. reported, that type IV collagen fragments in plasma of patients with abdominal aortic aneurysms was significantly higher than in healthy controls, while barely elevated in patients with peripheral artery disease (25). A larger scale study is needed to draw definite conclusions regarding the potential contribution of collagen type IV fragments from arteriosclerotic vessels to the circulation.

The study has some limitations. Data are based on those LITHE and RADIATE participants who consented to enter the present biomarker sub-study. However, the demographic and clinical profile of these subsamples compared well with the entire study populations presented by Kremer *et al*. (26) and Emery *et al*. (14). The studies were not designed to assess extra-articular pathologies involving basement membrane structural proteins, cardiovascular disease in particular. Anti-cyclic citrullinated protein antibody (ACPA) concentration, rheumatoid factor (RF) status, metalloproteinase involvement and pro-angiogenic mediators such as vascular endothelial growth factor (VEGF) were not determined, but may be relevant to compare against C4M in future studies. Major advantages of the study include the prospective design with inclusion of two large and well characterised cohorts of RA patients which yielded largely concordant results.

In conclusion, this study provides evidence that C4M, a novel serological marker of type IV collagen turnover, can be used as a biochemical adjunct for assessment of RA regarding disease activity, response to tocilizumab treatment and structural joint damage progression. Thus, C4M is a biologically plausible indicator of type IV collagen turnover which holds promise as a useful tool for disease mapping and monitoring of goal directed treatment at the molecular level.

## Acknowledgements

The study was supported by the Danish Research foundation and Institute of Clinical Research, the University of Southern Denmark (Odense, Denmark). Samples from the LITHE study were kindly donated by Roche-Genentech. We acknowledge that the clinical data were attained from patients who volunteered in the study, collected and supervised by the ACTEMRA group. Additionally, we appreciate the proofreading by Anna Dylander.

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