# Impact of short-term therapies with biologics on prothrombotic biomarkers in rheumatoid arthritis

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## ABSTRACT

**Background.** *Imbalance of haemostasis in patients with rheumatoid arthritis (RA) contributes to future risk of cardiovascular diseases (CVD). Prothrombotic molecules, e.g. fibrinogen, D-dimer, and tPA are elevated in plasma of RA patients, being associated to CVD. There is no imformation about the influence of biological drugs, e.g. anti-CD20 and tumor necrosis factor (TNF) antibodies on these prothrombotic molecules.*

**Objective.** *To assess whether anti-TNF and anti-CD20 therapies modify the profiles of cardiovascular risk factors in patients with RA.*

**Methods**. *The expression of prothrombotic molecules in plasma was investigated in 10 RA patients before and after treatment with TNF-alpha antibodies and in another 12 RA patients before and after anti-CD20 treatment.*

**Results**. *Both anti-TNF and anti-CD20 infusions gave rise to clear clinical improvement. However, only anti-CD20 infusion significantly ($p=0.05$) reduced concentration of fibrinogen ($p=0.05$), D-dimer ($p<0.001$), as well as tPA levels ($p<0.01$). In contrast, in TNF antibody treated patients only tPA levels were significantly decreased following the treatment ($p<0.05$).*

**Conclusions**. *Infusion of CD20 antibodies to the patients with active RA led to a clearly reduced plasma levels of predictors of CVD indicating that this treatment, apart from its anti-inflammatory properties, may reduce the risk for future CVD in RA.*

## Introduction

Would RA patients benefit from biological agents more than down regulation of joint inflammation? RA patients have an increased mortality and a shorter median survival than expected for the general population (1). This might be explained by increased rates of cardiovascular (CV) events (2). The increased incidence of CV events in RA patients is independent of traditional risk factors e.g. hypertension, smoking, and hypercholesterolemia, suggesting additional mechanisms (3-5). It has been established that the generalized inflammatory state that characterizes RA, may render these patients more prone to develop CVD, either directly, by accelerating atherosclerosis, or indirectly, by deteriorating cardiovascular risk factors (6-7). Systemic chronic inflammation, expressed as, *e.g.* CRP increase is strongly and consistently associated with the risk of future cardiovascular events (8). Several prothrombotic molecules, *e.g.* tissue plasminogen activator (tPA), fibrinogen, vWF, and fibrin d-dimer (9-10) are linked to the future risk of developing CV, and they were found significantly elevated in RA patients in comparison with general population (11). However, there is scarce information on the influence of biological therapies on cardiovascular risk factors in RA.

In the present study we assessed whether biological agents (infliximab and rituximab) would change the profiles of cardiovascular risk factors in RA. To this end, we assessed the plasma levels of tPA, fibrinogen, d-dimer, and plasminogen activator inhibitor-1(PAI-1) before and 3 months after the anti-CD 20 treatments or following 5 infusions of anti-TNF in 22 RA patients.

## Methods

*Patients*

Blood samples were collected from 22 patients with RA (5 males, 17 females, age range 27-72 years) attending the Rheumatology clinics, at Sahlgrenska University Hospital in Gothenburg, prior to for infusions of antibodies against TNF (infliximab, total dose 1000 mg, 5 infusions) or anti-CD20 (rituximab, 1000mg/infusion, two infusions, two weeks between each infusion). Clinical characteristics of RA patients are presented in Table I. All patients receiving rituximab had previously been treated with TNF-alpha blockers without satisfactory clinical improvement. The biologic drugs were discontinued at least 8 weeks before the first rituximab infusion. The Medical Ethics Committee at Göteborg University approved the study, and informed consent was obtained from all patients after written and verbal information had been given.

*Blood sampling*

Blood samples were obtained prior to anti-TNFα treatment, after the second

infusion, after the third, and after the fifth. For those patients receiving anti-CD20 treatment, blood was collected before and 3 months after the treatment. After centrifugation at 3500 rpm at 4°C for 15 minutes, plasma was collected and stored at -80°C until assayed.

*Measurements of prothombotic molecules*

The levels of tPA antigen, PAI-1 antigen, D-dimer, and fibrinogen were determined using a specific sandwich ELISA kits (Haemochrom diagnostica, Essen, Germany) according to the manufacturer's protocols. The minimum detection threshold was 0.5 ng/ml for tPA antigen and for PAI-1 antigen, 10ng/ml for D-dimer, and 2.5 ng/ml for fibrinogen.

*Statistical analysis*

Non-parametric methods were used for all statistical comparisons since data showed a skewed distribution. The Wilcoxon signed rank test for paired samples was used to compare differences between variables (DAS28, ESR, CRP, the levels of tPA antigen, PAI-1 antigen, D-dimer, and fibrinogen) before and after the treatments in all matched samples. Values are expressed as the median (25%-75% interquartile range). Spearman correlation test was used for assess the association of ΔCRP with Δ values of prothrombotic molecules. $P<0.05$ was considered significant in all the tests.

## Results

*Reduced clinical manifestations and activity of RA following anti-TNF and anti-CD20 treatments*

Before the treatment all patients displayed high disease activity of RA having median DAS28 5.8 (5.5-6.3) in rituximab group and 5.7 (4.7-5.9) in the infliximab group. After 3 months treatment with rituximab, DAS28 showed a significant decrease to 4.3 (3.9-4.8, $p<0.01$). Patients treated with 5 infusions of infliximab also had clear clinical improvement (DAS28 3.7 (3.5-4.7), $p<0.05$). The reduction in DAS28 was accompanied with changes in other inflammatory parameters such as a substantial decrease of ESR and C-reactive protein (Table I).

**Table I.** RA patient data.

| Parameter | Anti-CD20 treatment | Anti-TNF-α treatment |
|---|---|---|
| Number of patients | 12 | 10 |
| Male/female | 2/10 | 3/7 |
| Age, years | 61 (44-69) | 56 (27-72) |
| Erosive | 11 | 9 |
| Rheumatoid factor-positive | 10 | 8 |
| CRP (mg/l) | Before: 22 (15-34)<br>3 months: 0 (0-12) *** | Before: 41 (9-100)<br>5 infusions: 9 (8-34)* |
| ESR (mm/h) | Before: 36 (26-56)<br>3 months: 21 (11-32.5)** | Before: 44 (11-85)<br>5 infusions: 18 (12-44) |
| DAS28 | Before: 5.8 (5.5-6.3)<br>3 months: 4.3 (3.9-4.8)*** | Before: 5.7 (4.7-5.9)<br>5 infusions: 3.7 (3.5-4.7)* |
| Treatment in combination with biological agents: | | |
| MTX | 10 | 9 |
| Azathioprin | 2 | 1 |
| Cyclosporine | 3 | 0 |
| Dose of biological agents | 1000 mg x 2 | Total 1000 mg, 5 infusions |

Data are presented as median (25%-75%interquartile range). Wilcoxon signed rank test was performed to compare the differences of CRP, ESR, and DAS28 before and after treatments of anti-CD20 or anti-TNF. *$p<0.01$; **$p<0.05$; ***$p<0.001$.

*Impact of anti-TNF and anti-CD 20 treatments on hemostatic cardiovascular risk factors*

The plasma levels of pro-thrombotic molecules following the infliximab or rituximab treatments are presented in Figure 1. Significantly reduced plasma levels of fibrin D-dimer were found following the anti-CD20 treatment (1189μg/ml *vs*. 565μg/ml, $p=0.001$) (Fig. 1a). In contrast, the impact of infliximab on D-dimer seemed transient. Reduced D-dimer levels were only seen after 2 weeks (727μg/ml *vs*. 457μg/ml, $p<0.01$), later rising to the pretreatment levels (Fig. 1e).

Decreased tPA levels were also indentified in the RA patients after 3 months of rituximab treatment (9.6 ng/ml *vs*. 38.4ng/ml, $p<0.01$) (Fig 1b). In infliximab group, we also found substantial reduction of tPA levels after 5 infusions of infliximab treatment (20.6ng/ml *vs*. 11.2ng/ml, median, $p<0.05$) (Fig. 1f).

There was a tendency of decreased fibrinogen levels in RA patients following rituximab treatment (2.1mg/ml *vs*. 0.7mg/ml, median; $p=0.05$) (Fig. 2c), while fibrinogen levels were not significantly altered after infliximab treatment (Fig. 2g).

No significant difference was found regarding plasma levels of PAI-1 in any of the studied groups (Figs. 2d and 2h).

*Association of the changes of CRP with the changes of cardiovascular risk factors following anti-TNF and anti-CD 20 treatments*

To study the possible causes of reduction of plasma levels of pro-thrombotic molecules, we applied non-parametric correlation (Spearman test) to study the possible association between the changes of CRP (ΔCRP) and the changes of the plasma levels of the thrombotic molecules (Δ). Strong possitive correlation between ΔCRP and ΔD-dimer was found in the plasma from patients treated with anti-CD20 (r2=0.52, $p<0.001$, Fig. 2). In contrast, ΔtPA antigen and Δfibrinogen were not correlated to ΔCRP in the same group (not shown). In the patients receiving anti-TNF therapy, no correlations were found between ΔCRP and Δ plasma levels of any of pro-thrombotic molecules tested (data not shown).

## Discussion

In this study we show that although both anti-TNF and anti-CD20 had satisfactory clinical impact on disease activity of RA patients, a substantial reduction of plasma levels of pro-throm-

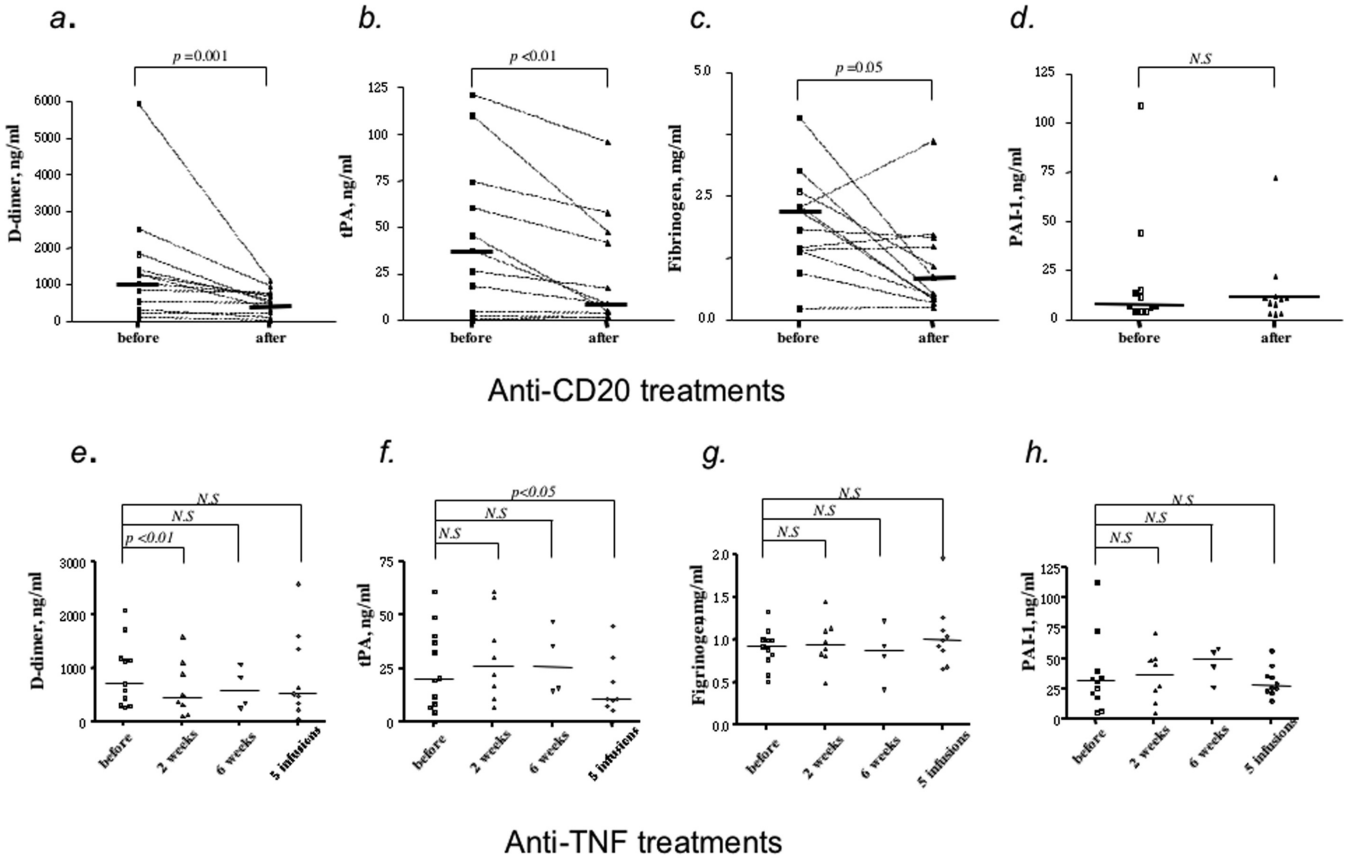


**Fig. 1.** The upper panel shows the concentration of D-dimer (a), tPA antigen (b), fibrinogen (c), and PAI-1 antigen (d) in 12 patients with active RA before and 3 months after treatment with a chimeric anti-CD20 monoclonal antibodies. The lower panel shows the concentration of D-dimer(e), tPA antigen (f), fibrinogen (g), and PAI-1 antigen (h) in 12 patients with active RA before and 2 weeks, 6 weeks, and 5 infusions after treatment with a chimeric anti-TNF-alfa monoclonal antibodies. Data are presented as individual values and median. *$p$<0.05, **$p$<0.01, ***$p$<0.001.

botic molecules (D-dimer, tPA, and fibrinogen) are only found in the patients treated with anti-CD20. Importantly, reduced D-dimer levels were strongly correlated to the suppressed inflammation (ΔCRP).

It is becoming increasingly clear that systemic inflammation, activation of the coagulation system, impaired physiological anti-coagulant pathway, and suppressed fibrinolytic system give rise to hemostatic imbalance with elevated pro-thrombotic molecules in circulation. Significant correlation between ΔCRP and ΔD-dimer suggests that at least reduced D-dimer is due to suppressed inflammation following anti-CD20 treatment. Recently, it has been reported that levels of D-dimer in RA patients were significantly decreased after 14 weeks of anti-TNF therapy (12). Agirbasli M *et al*. demonstrated a significant reduction of plasma PAI-1 with infliximab after 2 weeks, while tPA levels were unchanged (13). However, our study showed that in the patients that received anti-TNF antibodies, the only reduction was found regarding tPA levels after 5 infusions, and no difference was found in levels of fibrinogen and D-dimer. This discrepancy might be due to relatively limited patient number in both our study and previous studies. In assessing the short-term influence of the TNF-alpha blockade on some other non-traditional markers of atherosclerosis in RA, it was found that infusion of infliximab in a series of RA patients with severe disease, refractory to conventional therapy, yielded a rapid and dramatic improvement of insulin resistance and sensitivity (14). Also, following infliximab infusion, a reduction in the levels of some bio-

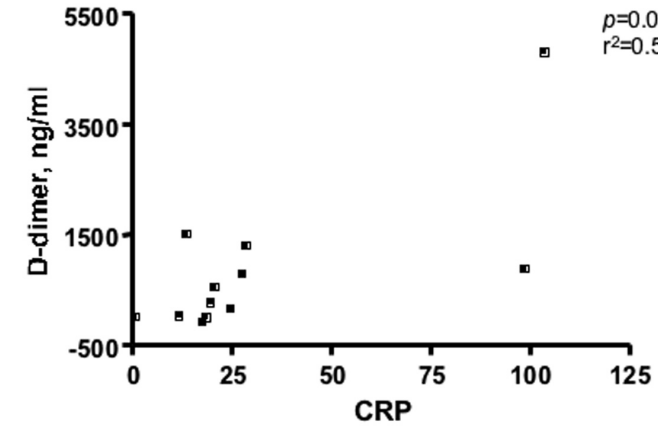


**Fig. 2.** The correlation of changes of CRP levels (ΔCRP) in plasma from RA patients after treatment of chimeric anti-CD20 monoclonal antibodies with the changes of D-dimer (ΔD-dimer) levels in plasma.

markers associated with atherogensis was found (15). Moreover, although no immediate effect of the administration of infliximab on adiponectin levels was observed, a dramatic decrease of resistin levels, which were found to have a strong correlation with CRP values, was observed when resistin levels obtained immediately after infusion of the drug were compared with those found immediately before infliximab infusion (16, 17).

What are the clinical implications of our finding? It has already been shown that the risk of a first CVD is lower in the patients with anti-TNF treatment (18). However, it is also true that the improvement of endothelial function in patients with RA following infliximab therapy is transient as values of flow-mediated endothelial dependent vasodilatations return to baseline levels 4 weeks after the administration of this drug (19), and progression of subclinical atherosclerosis after a 3-year median duration of treatment with infliximab has also been reported in RA patients with long standing severe RA (20). Due to this, we wondered whether the prolonged reduction of hemostatic predictors of cardiovascular risk observed by anti-CD20 therapy but not with infliximab therapy might be more effective to reduce the cardiovascular mortality observed in patients with this chronic inflammatory disease. However, long-term prospective studies are required to determine whether such findings translate into tangible improvements in cardiovascular morbidity and mortality in RA patients.

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