Prothrombotic biomarkers in patients with rheumatoid arthritis: the beneficial effect of IL-6 receptor blockade

R. Gualtierotti[1], F. Ingegnoli[1], S. Griffini[2], E. Grovetti[2], P.L. Meroni[1,3], M. Cugno[2]

[1]Dipartimento di Scienze Cliniche e di Comunità, Università degli Studi di Milano, Cattedra di Reumatologia, Istituto G. Pini, Milan, Italy; [2]Medicina Interna, Dipartimento di Fisiopatologia Medico-Chirurgica e dei Trapianti, Università degli Studi di Milano, Ospedale Maggiore Policlinico, Fondazione IRCCS Ca' Granda, Milan, Italy; [3]IRCCS Istituto Auxologico Italiano, Milan, Italy.

Abstract

Objective

The pro-inflammatory cytokine interleukin (IL)-6 is involved in the pathogenesis of both rheumatoid arthritis (RA) and cardiovascular events. We evaluated the correlation of prothrombotic biomarkers, in particular those of thrombin generation, with inflammatory and clinical parameters in RA patients treated with tocilizumab, an IL-6 receptor (IL-6R) inhibitor. Naïve and maintenance patients were compared.

Methods

We studied 15 RA patients undergoing tocilizumab infusions at a University Outpatient Clinic. Eight received tocilizumab for the first time and were evaluated at baseline. Seven were in maintenance therapy (9 to 77 months). All 15 patients were evaluated four weeks after the last administration of tocilizumab. At each time, we assessed disease activity score 28 (DAS28), erythrocyte sedimentation rate (ESR) and plasma levels of C-reactive protein (CRP), IL-6, soluble (s)IL-6R, tumour necrosis factor-alpha (TNF-alpha), prothrombin fragment F1+2 and fibrin fragment D-dimer. Forty healthy subjects served as basal controls.

Results

At baseline, RA patients showed a moderate-to-high disease activity and median ESR of 51 mm/1st hour (interquartile range 25–63). Plasma levels of CRP (p=0.0001), IL-6 (p=0.043), sIL-6R (p=0.003), TNF-alpha (p=0.0001), F1+2 (p=0.0001) and D-dimer (p=0.002) were higher than those of healthy controls. After four weeks we observed reduction of DAS28 (p=0.0001), ESR (p=0.0001), CRP (p=0.014), TNF-alpha (p=0.006), F1+2 (p=0.009) and D-dimer (p=0.04). No differences were observed between naïve and maintenance patients.

Conclusion

The reduction of prothrombotic biomarkers parallels the reduction of inflammatory parameters and clinical symptoms in RA patients treated with tocilizumab, both four weeks after the first administration and during maintenance therapy.

Key words

rheumatoid arthritis, tocilizumab, IL-6, inflammation, coagulation, prothrombotic biomarkers, cardiovascular risk

Roberta Gualtierotti, MD
Francesca Ingegnoli, MD
Samantha Griffini, PhD
Elena Grovetti, PhD
Pier Luigi Meroni, MD
Massimo Cugno, MD

Please address correspondence to:
Massimo Cugno, MD,
Medicina Interna,
Dipartimento di Fisiopatologia Medico-Chirurgica e dei Trapianti,
Università degli Studi di Milano,
Ospedale Maggiore Policlinico, Fondazione IRCCS Ca' Granda,
Via Pace 9,
20122 Milano, Italy.
E-mail: massimo.cugno@unimi.it

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Introduction

Rheumatoid arthritis (RA) is a chronic systemic inflammatory disease of unknown aetiology that affects up to 1% of the adult population (1). RA is characterised not only by persistent synovitis that causes destructive lesions, pain and functional disability, but also by systemic inflammation and autoantibody production (2). Patients with RA suffer from a chronic disabling disease, which is burdened by a high cardiovascular morbidity and mortality (3, 4). The pathogenic mechanisms of increased cardiovascular risk in patients with RA are complex and not fully explained by traditional cardiovascular risk factors such as smoking, hypertension and obesity (5, 6). Part of the risk may be due to the activation of coagulation which has been demonstrated in RA (7, 8). The close link between the coagulation system and inflammation, which is a hallmark of RA, has been known for some time (5, 9, 10).

Interleukin (IL)-6 is a key pro-inflammatory cytokine involved in RA pathogenesis (11) and elevated levels of IL-6 are independently associated with increased cardiovascular risk in the general population (12, 13). It is well known that pro-inflammatory cytokines such as IL-6 and tumour necrosis factor alpha (TNF-alpha) can induce the expression of tissue factor (TF), the main initiator of blood coagulation (14). TF activates the coagulation cascade with thrombin generation and formation of the fibrin clot. A reliable marker of thrombin generation *in vivo* is the prothrombin fragment F1+2 which is released from the amino terminus of the prothrombin molecule upon activation of factor X (15). Another biomarker of coagulation activation is the fibrin fragment D-dimer which is released from stabilised fibrin as a consequence of fibrinolysis activation (15). At the cellular level, coagulation factors interact with receptors called PAR (protease-activated receptor) inducing the expression of pro-inflammatory cytokines (16).

Tocilizumab is a humanised monoclonal antibody anti- IL-6 receptor (IL-6R) (17) which binds both membrane-bound and soluble forms of IL-6R (mIL-6R and sIL-6R, respectively) (18), thereby blocking IL-6-mediated signalling and inflammatory effects (18, 19). Tocilizumab is an effective and safe treatment in long-standing rheumatoid arthritis even when it is refractory to other biologic treatments (20) and its dosage can be tapered when a low disease activity is achieved (21). Among the side effects of tocilizumab particular concerns have been raised regarding the alteration of the lipidic profile (22). However observational studies failed to demonstrate an increase of thrombotic risk (23). Furthermore, a recent genetic study on a large scale has shown that a single nucleotide polymorphism (SNP) in IL-6R gene, which leads to a reduction of fibrinogen and C-reactive protein (CRP) levels, is associated with a lower risk of coronary events. A similar reduction of fibrinogen and CRP can be obtained in patients with RA treated with tocilizumab (24).

With this as background, we evaluated the correlation between plasma prothrombotic biomarkers, in particular those of thrombin generation, and inflammatory and clinical parameters in RA patients after treatment with tocilizumab. Naïve patients and patients during maintenance were compared and tocilizumab side effects on the lipid profile were assessed.

Patients and methods

Patients

The study involved 15 consecutive RA patients (13 women and two men) attending the Department of Rheumatology at Istituto Gaetano Pini, Milano, Italy, who fulfilled the revised American College of Rheumatology criteria for the classification of RA (25). Their demographic and clinical characteristics are reported in Table I. Their median age was 57 years and their median disease duration was 15 years. Eight patients were evaluated before tocilizumab therapy (baseline) during active disease as defined by a 28-joint disease activity score (DAS28) ≥3.2 (26). Seven were in maintenance therapy from a median time of 20 months (minimum 9 - maximum 77 months). All patients were evaluated four weeks after the last administration of intravenous tocili-

Table I. Demographic and clinical characteristics of the study population.

Total RA patients n=15	
Median age (min-max)	57 yrs (26-68 yrs)
Mean disease duration (min-max)	15 yrs (5-36 yrs)
Frequency (n)	
Active disease (DAS-28 ≥ 3.2)	53.3% (8)
Maintenance therapy	46.7% (7)
RF	87% (13)
ACPA	73% (11)
Daily equivalent prednisone dosage ≥ 5 mg/day	47% (7)
DMARDs	67% (5 MTX≥10 mg; 4 LEF 10-20 mg/day; 2 OHCQ 200 mg/day)
NSAIDs	87% (13)
Statins	27% (4)
Smoking habit	40% (6)

RA: rheumatoid arthritis; DAS-28: 28-joint disease activity score; RF: rheumatoid factor; ACPA: anti-citrullinated protein antibodies; DMARDs: disease-modifying anti-rheumatic drugs; MTX: methotrexate; LEF: leflunomide; OHCQ: hydroxychloroquine; NSAIDs: non-steroidal anti-inflammatory drugs.

zumab (8 mg/kg over 1 hour monthly). At baseline and four weeks after the last tocilizumab infusion we evaluated DAS28, biomarkers of inflammation (CRP, erythrocyte sedimentation rate [ESR], IL-6, sIL-6R and TNF-alpha) and prothrombotic biomarkers (F1+2 and D-dimer). At baseline and after 12 weeks we evaluated total cholesterol, high density lipoprotein (HDL), low density lipoprotein (LDL), triglyceride levels, haemoglobin level, white blood cell and platelet count, alanine transaminase (ALT) and aspartate transaminase (AST).

The control group consisted of 40 age- and sex-matched healthy subjects (35 women, five men) in the absence of active inflammation and history of thrombosis.

Blood sampling

Morning fasting blood samples were collected in vacutainer tubes (Vacuette Greiner bio-one) by means of clean puncture of an antecubital vein with minimal stasis, using sodium citrate 3.8% as anticoagulant. Blood collection was performed before infusion of tocilizumab (baseline) and four weeks thereafter. The samples were centrifuged at 2000 g, divided into aliquots, frozen and stored at -80° C until testing.

Written informed consent was obtained from all subjects and the study was approved by the Ethics Committee of the Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milan, Italy.

Biomarkers

C-reactive protein (CRP) concentration was measured using a sandwich ELISA (Zymutest CRP, Hyphen BioMed, Neuville-sur-Oise, France). Intra- and inter-assay coefficients of variation (CV) were smaller than 11%.

Interleukin-6 (IL-6) levels were measured in plasma by an ELISA (R&D Systems, Minneapolis, MN). Intra- and inter-assay CVs were 4.2% and 6.4%.

Soluble IL-6 receptor (sIL-6R) levels were measured in plasma by an ELISA (R&D Systems, Minneapolis, MN). Intra- and inter-assay CVs <8.6%.

Tumour necrosis factor-alpha (TNF-alpha) plasma levels were measured using a direct solid-phase immunoassay (Enzyme Amplified Sensitivity Immunoassay; EASIA Biosource, Flerus, Belgium); with intra- and inter-assay CVs of 8% and 10%.

Prothrombin fragment F1 + 2 levels were measured in plasma using an enzyme-linked immunosorbent assay (ELISA) (Enzygnost F1 + 2; Behring Diagnostics GmbH, Marburg, Germany); with intra- and inter-assay CVs of 5% and 8%.

Fibrin fragment D-dimer plasma levels were measured using a commercial ELISA (Zymutest D-dimer, Hyphen Biomed, Neuville sur Oise, France). Intra- and inter-assay CVs were 10% and 15%.

Statistical analysis

Descriptive statistics are reported as median and interquartile (IQ) range (25^{th} and 75^{th} percentiles). We used non-parametric tests for independent samples to evaluate differences between healthy controls and RA patients and for paired samples to evaluate the effect of treatment with tocilizumab in the same group. Significance level was set at $p<0.05$. Correlations were evaluated by Spearman non-parametric tests. Data were analysed using the SPSS PC statistical package, v. 22 (SPSS Inc., Chicago, IL).

Results

Active RA patients before tocilizumab therapy

Before starting tocilizumab therapy, RA patients showed moderate-to-high disease activity as demonstrated by a median value of DAS28 of 3.54 (interquartile range 2.92–4.33) (Fig. 1). Baseline median levels of ESR and plasma CRP were higher ($p=0.0001$) than healthy controls, 51 mm/1^{st} h (25–63 mm/1^{st} h) and 5.34 mg/ml (1.80–10.03 mg/ml) *vs*. 4 mm/1^{st} h (2-6 mm/1^{st} h) and 0.80 mg/ml (0.30–1.40 mg/ml) respectively (Fig. 1). Significantly higher levels were observed for IL-6 (median 14.98 pg/ml [IQ 5.48–49.90 pg/ml]) ($p=0.043$), sIL-6R (136841 pg/ml [60426–240141 pg/ml]) ($p=0.003$) and TNF-alpha (8.29 pg/ml [7.46–8.68 pg/ml]) ($p=0.0001$) than values of healthy controls: 8.00 pg/ml (6.00–10.00 pg/ml) for IL-6, 47872 pg/ml (36607–66200 pg/ml) for sIL-6R, and 1.27 pg/ml (1.00–1.88 pg/ml) for TNF-alpha, as shown in Figure 2.

F1+2 and D-dimer were significantly higher at baseline, 256 pmol/L (226-343 pmol/L) and 1194 pmol/L (413-1895 pmol/L), respectively, as compared to healthy controls, 157 pmol/L (116–180 pmol/L) and 193 pmol/L (130–374 pmol/L) ($p=0.0001$ and $p=0.002$ respectively), as shown in Figure 3.

RA patients in remission four weeks after the last tocilizumab infusion

Four weeks after the last administration of tocilizumab, there were no differences in the evaluated parameters between patients treated with tocilizumab for the first time and patients in maintenance therapy (Fig. 1-3). In both groups, clinical and laboratory parameters showed a significant improvement

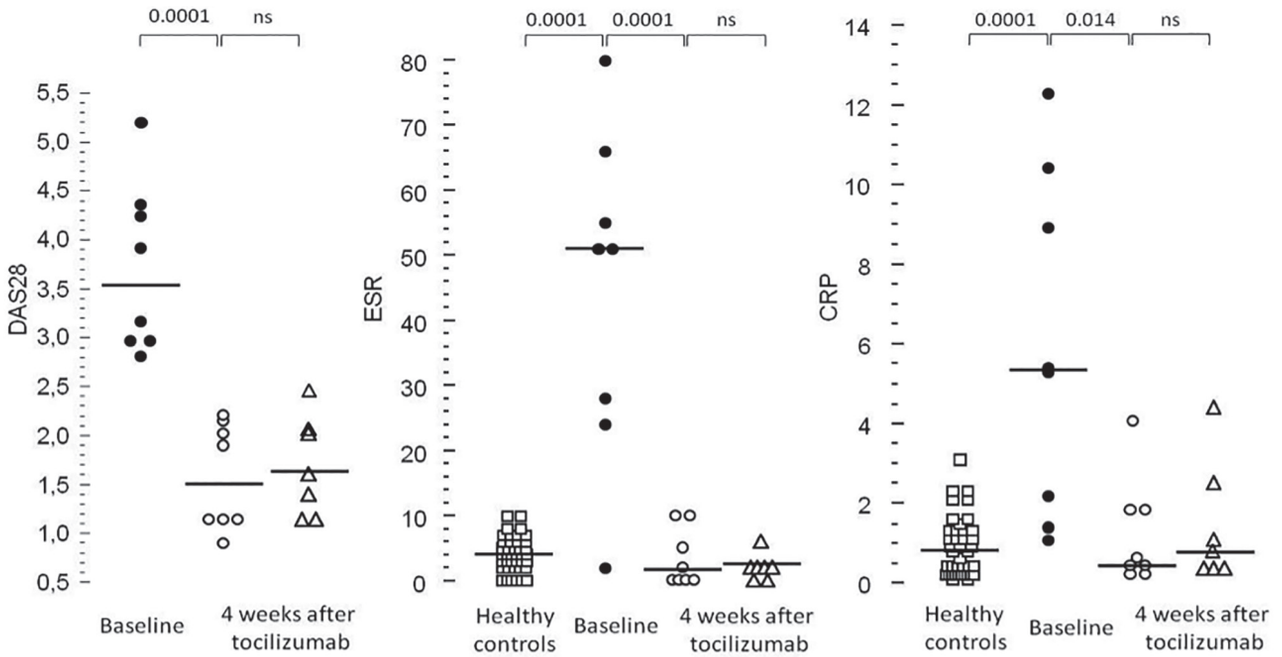

Fig. 1. Disease activity score on 28-joints (DAS-28), erythrocyte sedimentation rate (ESR) and serum levels of C-reactive protein (CRP) in patients with rheumatoid arthritis. Full circles represent patients never treated with tocilizumab (baseline). Empty circles represent patients studied after 4 weeks from the first administration of tocilizumab. Triangles represent patients studied after 4 weeks from the last administration of tocilizumab during maintenance therapy. Squares represent healthy controls. Horizontal lines represent median values.

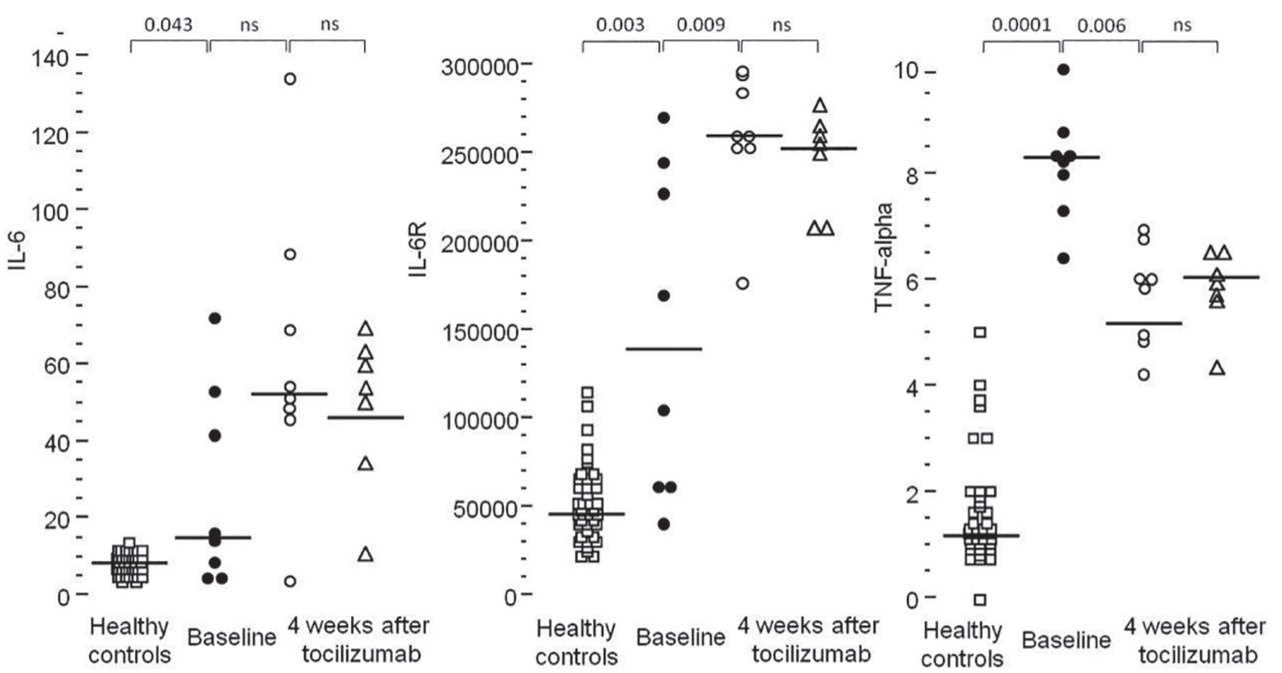

Fig. 2. Plasma levels of interleukin-6 (IL-6), interleukin-6 receptor (IL-6R) and tumour necrosis factor-alpha (TNF-alpha) in patients with rheumatoid arthritis. Full circles represent patients never treated with tocilizumab (baseline). Empty circles represent patients studied after 4 weeks from the first administration of tocilizumab. Triangles represent patients studied after 4 weeks from the last administration of tocilizumab during maintenance therapy. Squares represent healthy controls. Horizontal lines represent median values.

compared to baseline. In particular, in naïve patients we observed a reduction of DAS28 to 1.52 (1.15-2.05, p=0.0001), of ESR to 1.00 mm/1st h (0.00-8.75 mm/1st h, p=0.0001), of CRP to 0.50 mg/ml (0.36-2.19 mg/ml, p=0.014), of TNF-alpha to 4.94 pg/ml (4.49-6.82 pg/ml, p=0.006), of F1+2 to 179 pmol/L (96-248 pmol/L, p=0.009), and of D-dimer to 198 pmol/L (137-535 pmol/L, p=0.04). As expected, no reduction was observed in plasma levels of IL-6 (53.53 pg/ml [45.35-88.11 pg/ml]) and sIL-6R (258922 pg/ml [255143-292997 pg/ml]).

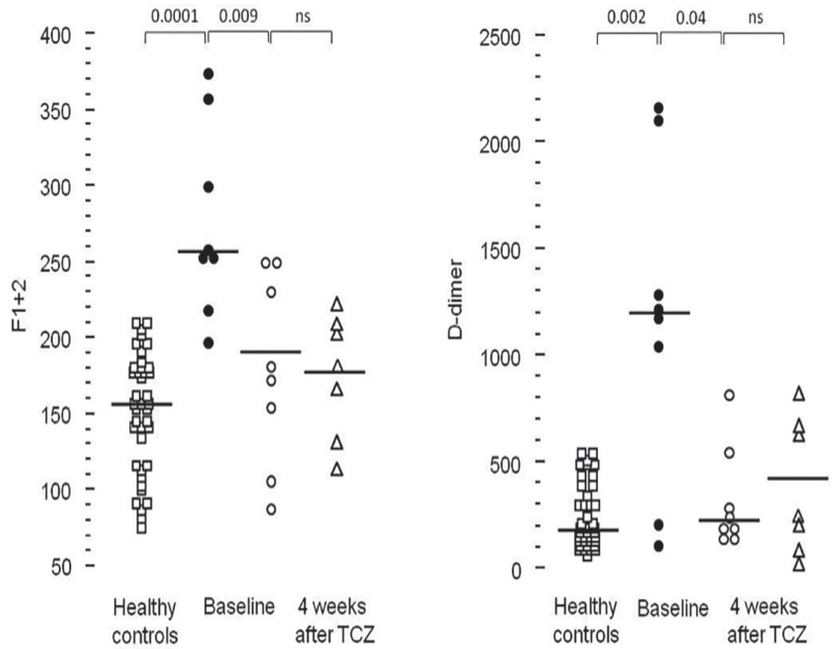

Fig. 3. Plasma levels of prothrombin fragment F1+2 and fibrin fragment D-dimer in patients with rheumatoid arthritis. Full circles represent patients never treated with tocilizumab (baseline). Empty circles represent patients studied after 4 weeks from the first administration of tocilizumab. Triangles represent patients studied after 4 weeks from the last administration of tocilizumab during maintenance therapy. Squares represent healthy controls. Horizontal lines represent median values.

Table II. Metabolic and haematologic effects on RA patients at baseline and after 12 weeks from the beginning of tocilizumab therapy.

Median (IQ range)	Baseline	After 12 weeks	*p*
Total Cholesterol mg/dl	220 (181-227)	241 (227-276)	0.012
HDL mg/dl	62 (56-85)	66 (60-86)	ns
LDL mg/dl	132 (118-154)	161 (136-186)	0.028
TGL mg/dl	128 (88-221)	151 (90-218)	ns
AST UI/L	21 (14-26)	28 (21-33)	0.012
ALT UI/L	20 (15-24)	33 (26-39)	0.017
Haemoglobin g/dl	12.10 (11.55-12.60)	13.00 (12.80-13.50)	0.049
Platelets n/mm^3	329500 (275750-370500)	221500 (199750-290750)	0.036
WBC n/mm^3	8750 (8125-10050)	5900 (5500-6925)	0.012
neutrophils n/mm^3	5060 (3275-6513)	2865 (2022-3875)	0.012

RA: rheumatoid arthritis; IQ: interquartile; ns: not significant; HDL: high-density lipoprotein; LDL: low-density lipoprotein; TGL: triglycerides; WBC: white blood cells; AST: aspartate transaminase; ALT: alanine transaminase.

Metabolic and haematologic effects of tocilizumab in RA patients

Because tocilizumab may have metabolic and haematologic side effects, we sought these effects in our patients after 12 weeks of tocilizumab treatment (Table II). Of note, a significant difference was found in total cholesterol and LDL compared to baseline. No significant difference was found between values after 12 weeks and at baseline for HDL and triglycerides.

After 12 weeks of tocilizumab treatment, we found a slight, but significant elevation of AST and ALT as well as a significant reduction in the white blood cell (WBC) and neutrophil counts. None of the patients included in our study experienced neutropenia even during maintenance therapy.

Haemoglobin levels and platelet number baseline were also affected by tocilizumab therapy with an increase of the former, and a reduction of the latter. No difference was found between patients in maintenance therapy and patients who had never undergone tocilizumab therapy.

Clinical and laboratory correlations in RA patients

We investigated whether correlations exist between disease activity and laboratory markers (Table III). As expected, DAS-28 correlated strongly with CRP and ESR values (r=0.68, p=0.001; r=0.82, p=0.001), with TNF-alpha levels (r=0.63, p=0.001), with F1+2 (r=0.72, p=0.001) and with D-dimer (r=0.68, p=0.001). F1+2 and D-dimer correlate each other (r=0.52, p=0.001). Furthermore, during the course of therapy we observed no cardiovascular events nor venous thromboses.

Discussion

This study showed the normalisation of inflammatory and prothrombotic biomarkers 4 weeks after tocilizumab infusion both in patients never treated before and in patients in maintenance therapy. At the same time, all patients were in clinical remission.

Therapy with tocilizumab is approved for the treatment of patients with moderate to severe RA, both in combination with conventional disease-modifying anti-rheumatic drugs (DMARDs) (27, 28) and in monotherapy (29). In our study, inflammatory and prothrombotic biomarkers were elevated at baseline and rapidly decreased during tocilizumab therapy. In RA patients, the increased risk of thrombotic diseases is associated with high levels of prothrombotic biomarkers (5, 30). By reducing them, a reduction of cardiovascular risk is expected. Indeed, according to a study that evaluated the safety of tocilizumab in RA patients, the incidence of cardiovascular events was much lower in patients receiving tocilizumab than in patients treated with other DMARDs (23). To the best of our knowledge, our study is the first showing a reduction of F1+2 plasma levels as an effect of tocilizumab therapy, in fact our patients during active disease had very high levels of F1+2 which normalised 4 weeks after the drug infusion. Since F1+2 is a reliable prothrombotic marker (15), its reduction is certainly beneficial as also previously demonstrated in RA patients after TNF-alpha blockade . We did not address the mechanisms responsible for the reduction of prothrombotic bio-

Table III. Correlations between disease activity and markers of inflammation, coagulation and fibrinolysis in 15 patients with rheumatoid arthritis on 23 oservations (8 at baseline and 15 after tocilizumab treatment).

	DAS-28	CRP	ESR	TNF	F1+2	D-dimer
DAS-28	1	0.68**	0.82**	0.63*	0.72**	0.68**
CRP	0.68**	1	0.59**	0.51*	0.45**	0.58**
ESR	0.82**	0.59**	1	0.65**	0.48**	0.60**
TNF	0.63*	0.51*	0.65**	1	0.58**	0.57**
F1+2	0.72**	0.45**	0.48**	0.58**	1	0.52**
D-dimer	0.68**	0.58**	0.60**	0.57**	0.52**	1

DAS-28: 28-joint disease activity score; CRP: C-reactive protein; ESR: erythrocyte sedimentation rate; TNF: tumour necrosis factor-alpha; F1+2: prothrombin fragment F1+2; D-dimer: fibrin fragment D-dimer. Values represent Spearman coefficients of correlation. *p=0.01; **p=0.001.

markers, which however are consistent with the observation that IL-6 blockade abrogates TF-dependent thrombin generation in experimental models (14). Furthermore, the association of an IL6R variant with lower risk of coronary heart disease indicates that the blockade of IL-6R may be beneficial (24). The reduction of F1+2 obtained after tocilizumab is similar to that obtained with infliximab (10). Other prothrombotic biomarkers such as fibrinogen and D-dimer were found to be rapidly reduced after IL-6R blockade in RA patients in a study by McInnes *et al*. (31). Makrilakis *et al*. further confirmed an antithrombotic effect of tocilizumab therapy as demonstrated by the parallel reduction of chemerin (an adipokine involved in inflammation) and plasminogen activator inhibitor-1 (an important inhibitor of the fibrinolytic system) (32). Our data are not sufficient to say that the decrease of prothrombotic markers is specifically related to IL-6 blockade, however, in RA patients, tocilizumab (acting on IL-6) seems to be more effective in reducing D-dimer levels than infliximab (acting on TNF-alpha), infact D-dimer levels were reduced of about 83% by tocilizumab (present study) as compared to 56% by infliximab (10).

The main side effects of tocilizumab therapy are a rise in the total cholesterol, LDL and triglyceride levels, reduction of white blood cell and platelet count, raise in haemoglobin levels and elevation of liver enzymes (23).

We found a significant elevation in total cholesterol and in LDL cholesterol in our patients treated with tocilizumab in agreement with other authors (27-29, 33). One possible explanation is the reduction of the systemic inflammatory state, which is also observed in patients treated with anti-TNF-alpha agents, who also experience an increase in total cholesterol and in triglyceride levels (34). However, the magnitude of the lipid changes associated with tocilizumab exceeds those following treatment with other potent anti-inflammatory regimens (35). These changes are typically evident within the 6th week of treatment with tocilizumab and remain stable during long-term therapy (22). Despite the lipid abnormalities observed during tocilizumab therapy, the incidence of cardiovascular events is not increased and even seems reduced as compared to RA population in an observational study (23). This may be in agreement with the observation that IL-6 blockade is able to induce a reduction in Lpa levels (36), a reduction of apolipoprotein a expression and the inhibition of IL-6-induced Lpa mRNA expression (37). In RA patients HDLs have reduced anti-inflammatory and anti-oxidant effects (38, 39). Tocilizumab therapy may revert these abnormalities. McInnes *et al*. found a raise in HDL small particles, thus suggesting a remodelling of HDL particles from a pro-inflammatory to an anti-inflammatory phenotype (31), as small HDL particles may be more active in cholesterol efflux and anti-inflammatory functions. In agreement we found a raise in HDL levels, although not statistically significant (Table II). Another explanation may regard cell cholesterol efflux, the first step of reverse cholesterol transport. This is a process leading to the biliary excretion of cholesterol in the intestine (40), and a reduction in the formation of the atherogenic plaque. Both traditional and biological DMARDs may enhance cell cholesterol efflux by means of the reduction of inflammation (41).

Our patients, after tocilizumab therapy, experienced a significant reduction of WBC count and in particular a reduction of neutrophils, which, however, remained within the normal range. Moreover, we observed a reduction of platelet count. Both WBC and platelet alterations have been reported after tocilizumab therapy (42) and may be a consequence of the blockade of the signalling pathways of IL-6 after the binding of tocilizumab to both mIL-6R and sIL-6R (43).

The raise in haemoglobin, which we observed in our patients treated with tocilizumab, is also a predictable beneficial side effect of tocilizumab which can be explained by a reduced expression of hepcidin as a consequence of the reduced chronic inflammatory state (44).

After tocilizumab, our patients showed a slight increase in AST and ALT (Table II) in accordance with other authors (45), however, none of our patients needed reduction or withdrawal of the drug for transaminase increase.

Apparently in contrast with the reduction of the inflammatory biomarkers after IL-6R blockade, IL-6 and sIL-6R were increased in our RA treated patients, as previously reported by Nishimoto *et al*. (19). They showed that, after tocilizumab infusion in RA patients, sIL-6R increased because its elimination half-life was prolonged by the formation of tocilizumab/sIL-6R complexes, and that free serum IL-6 increased because IL-6R–mediated consumption of IL-6 was inhibited by the unavailability of tocilizumab-free IL-6R. Similar conclusions were drawn by Uchiyama *et al*. which observed a marked increase in blood IL-6 and IL-6R levels after a single injection of tocilizumab in monkeys with collagen-induced arthritis (46). They demonstrated that the increased blood levels of IL-6 and IL-6R were due to a reduction of their clearance and not via the induction of their production. The normalisation of serum CRP, observed in our RA patients after tocilizumab, demon-

strates that IL-6 signalling is inhibited downstream even in the presence of increased levels of IL-6 and sIL-6R.

A limitation is the uncontrolled open-label design of the study which enrolled a limited number of active RA patients. However, this limitation may be counterbalanced by clear-cut differences indicating that the control of inflammation by tocilizumab is important in downregulating prothrombotic biomarkers.

In RA patients, tocilizumab showed to be effective in improving clinical symptoms and reducing inflammatory and prothrombotic biomarkers. These effects are already evident four weeks after the first administration of the drug and persist during maintenance therapy. Although our data must be confirmed by long-term studies on larger case lists, our observations of reduction of thrombin generation provide encouraging evidence that tocilizumab therapy could reduce the thrombotic risk in RA patients.

Key messages

- In rheumatoid arthritis patients, tocilizumab induces a parallel reduction of clinical symtpoms and thrombin generation.
- Tocilizumab beneficial effects are comparable in naïve patients after 4 weeks and in maintenance patients.
- Tocilizumab side effects on lipidic profile may be counteracted by the reduction of prothrombotic factors.

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