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### Dynamics of TNF during TNF inhibitor treatment

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# **The effect of methotrexate on tumor necrosis factor concentrations in etanercept- treated rheumatoid arthritis patients**

Rheumatology (Oxford) 2020

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## Abstract

**Objectives** Recently, we demonstrated that early low concentrations of circulating, adalimumab-bound TNF in RA patients treated with adalimumab was associated with future anti-drug antibody formation. Furthermore, low TNF was associated with less frequent baseline methotrexate (MTX) use. This is remarkable, because of the anti-inflammatory effects of MTX and a potential inhibiting effect on cytokine production. We hypothesized an indirect effect of non-MTX use on low TNF concentrations via immunogenicity. To investigate the effect of MTX on TNF concentrations independent of anti-drug antibody formation, we measured TNF in RA patients treated with etanercept, a drug with low immunogenicity.

**Methods** TNF was quantified in 186 consecutive etanercept-treated RA patients at baseline and at weeks 4, 16 and 28. The dynamics of TNF during etanercept treatment were compared with dynamics recently published for adalimumab.

**Results** We demonstrated that TNF concentrations at week 4 did not associate with baseline MTX or remission after 28 weeks. Furthermore, median (interquartile range) TNF increased from <112 (<112–<112) pg/mL at baseline to 548 (344–688) pg/mL at week 4 and remained stable at week 16 and 28 [598 (442–756) and 568 (444–755) pg/mL, respectively].

**Conclusion** Circulating TNF did not associate with MTX usage in etanercept-treated patients. This implies that MTX does not have a direct effect on TNF concentrations in circulation and that the association between early low TNF and non-use of MTX for adalimumab is thus most likely due to anti-drug antibody formation.

## **Introduction**

Numerous studies have investigated TNF concentrations during TNF inhibitor treatment.<sup>1-6</sup> However, these studies did not take into account that during treatment TNF is in complex with the TNF inhibitor, which interferes with the quantification of TNF. Consequently, the measured TNF will be underestimated.<sup>3,7</sup> Recently, our group developed a new drug-tolerant assay to quantify TNF concentrations in adalimumab-treated RA patients.<sup>7</sup> Circulating TNF was found to increase upon treatment, which may be explained by a prolonged TNF half-life, due to its tight binding to the antibody adalimumab, which has a very long half-life. As expected, TNF was biologically inactive, because the high amounts of TNF were essentially completely in complex with adalimumab. After the initial increase, longitudinal TNF concentrations were constant over time. However, low TNF concentrations at week 4 were associated with future anti-drug antibody (ADA) formation and less frequent remission after 52 weeks. The relationship between low TNF and ADA formation was validated and confirmed in a second independent cohort and in healthy volunteers who had received a single dose of adalimumab. Remarkably, patients treated with methotrexate (MTX) had significantly higher TNF concentrations at week 4 than patients without MTX treatment. MTX is an immunosuppressant that not only affects ADA formation,<sup>8</sup> but is also suggested to inhibit pro-inflammatory cytokine production (reviewed in 9). We hypothesize that MTX affects ADA formation against adalimumab, which in turn is associated with early low TNF concentrations and less frequent remission. This highlights the importance of ADA measurement during TNF inhibitor treatment.

The effect of MTX on TNF concentrations, independent of ADAs, can be investigated in more detail in etanercept-treated RA patients, since etanercept is essentially a non-immunogenic TNF inhibitor. Some studies demonstrated immunogenicity, albeit of low incidence, low titre and transient ADA responses, which do not target the idiotype.<sup>10,11</sup> We quantified TNF concentrations during 28 weeks in 186 etanercept-treated RA patients and studied the relation with clinical response and drug concentrations.

## **Materials and Methods**

### **TNF quantification with a drug-tolerant competition ELISA**

Adaptations to the drug-tolerant competition ELISA, as previously described for adalimumab,<sup>7</sup> were made in order to quantify TNF concentrations during etanercept treatment (Supplemental Figure S1C). Serum samples were diluted 10-fold in high performance ELISA (HPE) buffer (Sanquin Reagents, Amsterdam, The Netherlands)

supplemented with 1 mg/mL IVIg, to minimize non-specific binding (Nanogam, Sanquin, The Netherlands) (HPE+). Subsequently, serum was diluted 1:1 with biotinylated high-affinity adalimumab mutant antibody<sup>7,12</sup> (1 µg/mL in HPE+ buffer) in round-bottomed plates, resulting in a 20-fold serum dilution and 0.5 µg/mL high-affinity adalimumab mutant as final concentrations in the assay. A serially 2-fold diluted calibration curve of TNF in HPE+ buffer, which was calibrated against the World Health Organization standard, was also incubated 1:1 with biotinylated high-affinity adalimumab mutant antibody (1 µg/mL; final concentration in the assay 0.5 µg/mL). After overnight incubation at 37°C, 100 µL of sample and TNF calibration curve were incubated in Nunc MaxiSorp 96-well flat-bottomed plates (Thermo Scientific, Roskilde, Denmark), which were coated with monoclonal mouse anti-human TNF (3 mg/mL; clone 7, Sanquin Reagents, Amsterdam, The Netherlands) on a shaker platform for 2 h at 37°C. After washing five times with PBS containing 0.02% Tween 20, plates were incubated with 100 µL of streptavidin poly-horseradish peroxidase (1:10 000 dilution in HPE buffer) for 25 min at room temperature on a shaker platform. Plates were washed five times with PBS-0.02% Tween 20, and 100 µL of tetramethylbenzidine substrate (100 µg/mL) and 0.003% (v/v) hydrogen peroxide (Merck Millipore, Darmstadt, Germany) in 0.11 M sodium acetate buffer (pH 5.5) was added to each well. One hundred microlitres of 2 M H<sub>2</sub>SO<sub>4</sub> (Merck Millipore, Darmstadt, Germany) was added to stop the reaction. The optical density was measured at 450 nm and 540 nm with a plate reader (Synergy 2, Bio Tek Instruments). TNF concentrations were calculated with the TNF calibration curve. A cut-off was determined as the mean (3 SD) of healthy donor sera (n = 35).

### **Measurement of etanercept concentration and anti-etanercept antibody titre**

Etanercept concentrations were measured at trough, prior to the next etanercept dose, with ELISA, as previously described.<sup>13,14</sup> Briefly, microtiter plates were coated overnight with mouse monoclonal anti-human TNF (clone 5, Sanquin Reagents, Amsterdam, The Netherlands), followed by incubation with recombinant TNF $\alpha$  for 1 hour. Subsequently, bound etanercept in serially diluted patient sera was detected with biotinylated polyclonal etanercept-specific rabbit-anti idiotypic antibody. Etanercept concentrations were calculated with an etanercept titration curve. The lower limit of detection for this assay was 0.1 µg/mL.

Etanercept concentrations were previously manually measured. Meanwhile, the ELISA protocol was automated and re-validated. The old, manual version of the assay showed a good correlation with the automated assay, and a small systematic bias.<sup>15</sup> The data generated with the manual assay was corrected for this bias.

Anti-etanercept antibodies were measured previously with a bridging ELISA and a radio

immune assay, but no antibodies were detected in any of the sera.<sup>13</sup>

### **Patients**

A total of 197 consecutive biologic-naïve RA patients were enrolled in this prospective observational cohort study between December 2004 and November 2008 (Dutch Trial Register, NL6698). These patients have been described previously.<sup>13,14</sup> Patients were treated with a standard dose etanercept of 50 mg s.c. once every week or 25 mg s.c. twice weekly. Patients who did not start etanercept treatment at week 4 (n = 6) or patients without samples available for TNF measurements (n = 5) were excluded. Blood samples were drawn at baseline (week 0) and 4, 16 and 28 weeks after initiation of etanercept treatment at trough, and stored at -20°C until TNF concentrations were measured.

Patients were followed at the Amsterdam Rheumatology and immunology Center, Reade, Amsterdam, the Netherlands, and gave written informed consent. Patients fulfilled the ACR 1987 revised criteria for RA<sup>16</sup> and had active disease at the start of etanercept treatment, in agreement with the Dutch consensus statement on the initiation and continuation of TNF blocking therapy in RA. This study was approved by the Slotervaart Hospital and Reade Medical Research Ethics Committee.

RA patients treated with adalimumab, who were previously described in Berkhout *et al.*,<sup>7</sup> were also included in this study.

### **Clinical outcomes**

Clinical and laboratory assessments were scheduled at baseline and 4, 16 and 28 weeks thereafter and comprised: tender joint count, swollen joint count, patient's assessment of pain (visual analogue scales 0–100 mm), patient's global assessment of disease activity (visual analogue scales 0–100 mm), physician's global assessment of disease activity (visual analogue scales 0–100 mm), ESR, CRP, current medication use and HAQ. At baseline the following additional variables were recorded: age, gender, height, weight, duration of disease, IgM RF and ACPA status, medication history regarding prior and current DMARD therapy, glucocorticoid and previous TNF inhibitor use.

### **Statistical analysis**

The relationship between week 4 TNF concentration and Simplified Disease Activity Index (SDAI) remission or CRP at baseline and after 28 weeks was analyzed with a Spearman's rank correlation test. Last observation carried forward was used for SDAI scores for those patients that discontinued etanercept treatment prior to week 28. The association

between TNF concentrations at week 4 and MTX use at baseline was tested with a Mann-Whitney U test. Seven patients without detectable etanercept (<0.1 mg/mL) at week 4 were excluded from this analysis: four patients due to (temporary) discontinuation of etanercept treatment and three due to unknown reasons.<sup>14</sup> Deciles were formed according to etanercept or adalimumab concentrations at week 4 and median (interquartile range) TNF concentration was plotted for each decile. For all analyses, SPSS for Windows version 23.0 or GraphPad Prism version 8.0.2 were used. A P-value <0.05 (two-sided) was considered significant.

## Results

### **TNF concentrations quantified with a drug-tolerant assay**

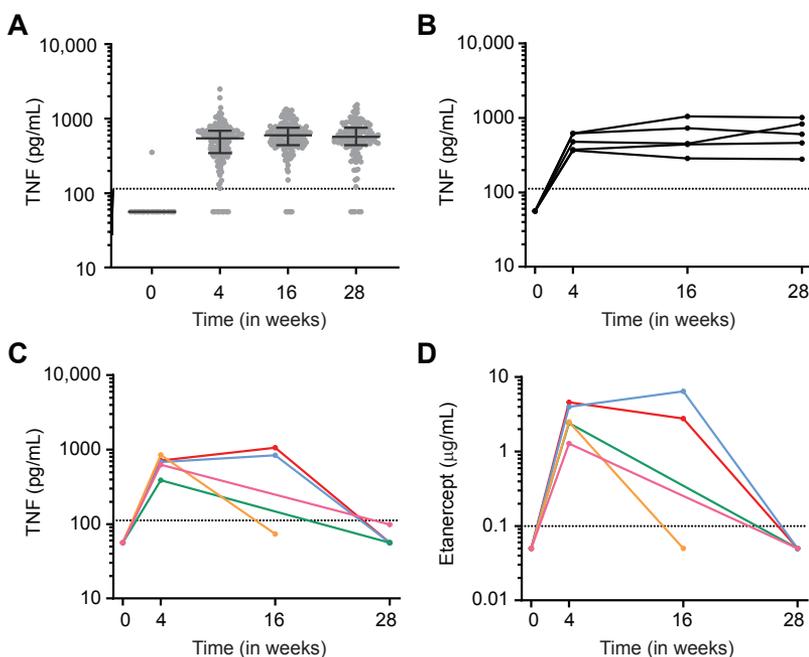
Since interference of etanercept in a conventional TNF ELISA is limited, but still observed (Supplemental Figure S1A), we first developed a drug-tolerant competition ELISA. This assay resulted in quantitative recovery of TNF in the presence of an excess of etanercept (Supplemental Figure S1, B and C). We used this assay to quantify TNF in 186 RA patients starting etanercept treatment (baseline characteristics; Supplemental Table S1). Baseline TNF was low, but substantially increased upon etanercept treatment (Figure 1A). Median TNF (interquartile range) was constant over 28 weeks in most patients [548 (344–688), 598 (442–756) and 568 (444–755) pg/mL at week 4, 16 and 28, respectively; Figure 1B]. This is also apparent when comparing the ratio between TNF at week 4 and 28, which is close to 1 (1.11; Supplemental Figure S2A).

Previously, we observed a more gradual increase in TNF in adalimumab-treated patients, with median (interquartile range) TNF of 146 (33–270), 199 (76–391) and 292 (133–506) pg/mL at week 4, 16 and 28, respectively.<sup>7</sup> Here, the ratio of week 4/28 is substantially higher than 1 (1.80; Supplemental Figure S2A), indicating that TNF levels increase after week 4. The ratio of week 16/28, on the other hand, is close to 1 for both etanercept and adalimumab (0.97 and 1.24, respectively; Supplemental Figure S2B), indicating that steady-state is reached at week 16.

TNF could no longer be quantified in patients in whom etanercept became undetectable (Figure 1, C and D). Furthermore, we observed a clear correlation between etanercept and TNF concentrations only in the low etanercept range (below ~1 mg/mL). This resembles the observations made for adalimumab (Supplemental Figure S3, A and B, respectively, and Ref. 7). For both etanercept and adalimumab, no clear correlation between drug levels and TNF concentrations is apparent above ~1 mg/mL.

**Week 4 TNF concentrations do not associate with MTX use**

Next, we investigated week 4 TNF concentrations in relation to baseline MTX use. At week 4, serum samples were available from 177 (95%) patients. As etanercept must be present for quantitative capture of TNF, we excluded patients with undetectable etanercept at week 4 [n = 7; of which 6 used MTX (see Methods for reasons undetectable etanercept)]. We did not observe a significant difference in week 4 TNF concentrations between patients who used MTX [566 (377–690) pg/mL] compared with patients that did not use MTX [478 (318–685) pg/mL; P = 0.32; Figure 2A]. Also, baseline prednisone use had no effect on week 4 TNF concentrations (P = 0.17 and 0.27 for adalimumab and etanercept, respectively).

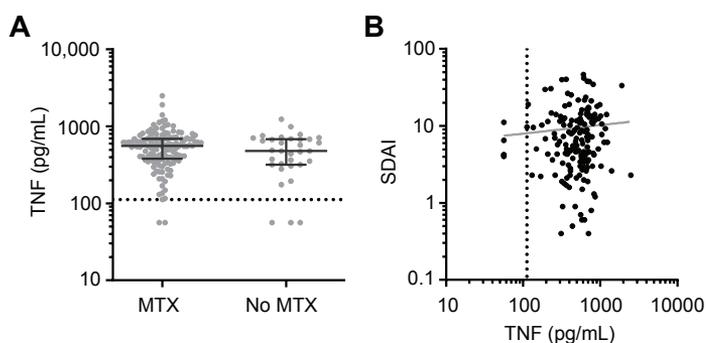


**Figure 1. TNF concentrations in etanercept-treated RA patients.** (A) Serum TNF concentrations at baseline (week 0), and 4, 16 and 28 weeks after etanercept initiation in 186 patients with RA. Each dot represents mean TNF concentration of a duplicate measurement in an individual patient; black lines show the median (interquartile range). (B) Representative examples of patients (n = 5) with longitudinal stabilized TNF concentrations. Representative examples (n = 5) of patients with diminished TNF concentrations over time (C) coinciding with undetectable etanercept (D). Colored lines in (C) correspond to patients with similar colored lines in (D). Dotted lines represent cut-off of TNF or etanercept (112 pg/mL or 0.1 µg/mL, respectively).

**Week 4 TNF concentrations do not associate with clinical response**

Under the assumption that TNF concentrations shortly after treatment initiation could be a reflection of the amount of inflammation, we investigated the relationship between

week 4 TNF concentrations and baseline disease activity. However, TNF concentrations at week 4 did not associate with baseline disease activity, according to SDAI or CRP levels (Spearman's  $\rho = 0.047$ ,  $P = 0.54$ ; Spearman's  $\rho = 0.055$ ,  $P = 0.47$ ; Supplemental Figure S4, A and B, respectively). Also at week 28 and 52, no association was found between SDAI remission and week 4 TNF concentration (Spearman's  $q = 0.096$ ,  $P = 0.22$ ; Figure 2B and Spearman's  $q = 0.064$ ,  $P = 0.41$ , data not shown, respectively), indicating that early TNF concentrations do not have predictive value for treatment response. Furthermore, RF and ACPA status were not associated with TNF concentrations at week 4 ( $P = 0.35$  and  $0.79$ , respectively, for adalimumab;  $P = 0.54$  and  $0.65$ , respectively, for etanercept).



**Figure 2. Relation between week 4 TNF concentrations and MTX use or clinical response.** (A) Week 4 TNF concentrations stratified by concomitant MTX use at baseline. Each dot represents mean TNF concentration of a duplicate measurement in an individual patient; black lines show the median (interquartile range).  $P = 0.32$ , Mann–Whitney U test. (B) Correlation between week 4 TNF concentrations and disease activity according to SDAI, at week 28 [Spearman's  $\rho = 0.096$ ,  $P = 0.22$  ( $n = 170$ )]. Grey line indicates log-log linear fit, weight by  $1/Y^2$ . (A and B) Dotted lines indicate cut-off of TNF (112 pg/mL). Patients without detectable etanercept ( $<0.1$  mg/mL) at week 4 were excluded from the analysis ( $n = 7$ ). SDAI: Simplified Disease Activity Index.

## Discussion

Recently, we demonstrated that adalimumab-treated RA patients with low circulating TNF concentrations at week 4 more often had detectable ADA formation during the 52-week follow-up, and that low circulating TNF was associated with a lower likelihood of remission. Furthermore, early low TNF was associated with less frequent baseline MTX use.<sup>7</sup> Unlike adalimumab, etanercept is essentially non-immunogenic. Here, we investigated the effect of MTX on TNF concentrations for etanercept, excluding immunogenicity as confounding factor. We demonstrated that TNF concentrations at week 4 did not associate with baseline MTX use, baseline CRP, or SDAI at 28 and 52 weeks. This implies that MTX does not have a direct effect on TNF concentrations and that the association between

early low TNF and MTX in adalimumab-treated RA patients is most likely due to antibody formation. Together, these findings also indicate once more that TNF in circulation (which is in complex with the drug, and biologically inactive) is not a reflection of inflammation or disease activity.<sup>7</sup>

Several studies to date have investigated TNF concentrations during etanercept treatment, albeit without assays optimized for TNF quantification in the presence of drug.<sup>3-6</sup> Only one small study (n = 6) investigated longitudinal TNF concentrations during etanercept treatment.<sup>6</sup> Two other studies indicate that increased TNF concentrations before week 6 predicted long-term efficacy of etanercept in JIA patients or in seronegative RA patients, but did not take etanercept concentrations into account.<sup>4,5</sup>

Interestingly, steady-state TNF concentrations during etanercept treatment were twice as high as in adalimumab-treated patients.<sup>7</sup> This points to a difference in clearance of drug-TNF complexes, perhaps due to the different number of Fc tails in the 1:1 etanercept-TNF complexes in comparison with the 3:1 adalimumab-TNF complexes.<sup>7,17</sup> Furthermore, we observed a more gradual increase in TNF during adalimumab treatment, while during etanercept treatment TNF concentrations reached steady state already at week 4. Since adalimumab-treated RA patients with low TNF concentrations at week 4 more often had detectable ADA formation during the 52-week follow-up, we speculate that this difference could be explained by the lack of ADAs in etanercept-treated patients, since antibody mediated clearance of etanercept-TNF complexes does not play a role.

Some patients had undetectable etanercept in serum at one, or multiple, visits accompanied by undetectable TNF, demonstrating that for quantitative capture of TNF a small amount of etanercept is required. We cannot precisely determine a cut-off etanercept concentration, since the number of data points in the low etanercept range is limited. Of note, undetectable etanercept cannot be explained by ADAs, but rather by the fact that patients (temporarily) discontinue etanercept treatment, for example due to medical reasons or treatment failure.<sup>14</sup>

Overall, we demonstrated that longitudinal TNF concentrations in etanercept-treated RA patients do not associate with baseline MTX use and clinical response. The association between early low TNF and non-use of MTX in adalimumab treatment is thus most likely due to ADA formation. Concomitant MTX use may reduce ADA formation to immunogenic TNF inhibitors. This study implies that circulating TNF concentrations measured during anti-TNF treatment are not reflective of (suppressed) inflammation, and advances our understanding of the mechanism of action of current anti-TNF treatments in relation to

clinical response.

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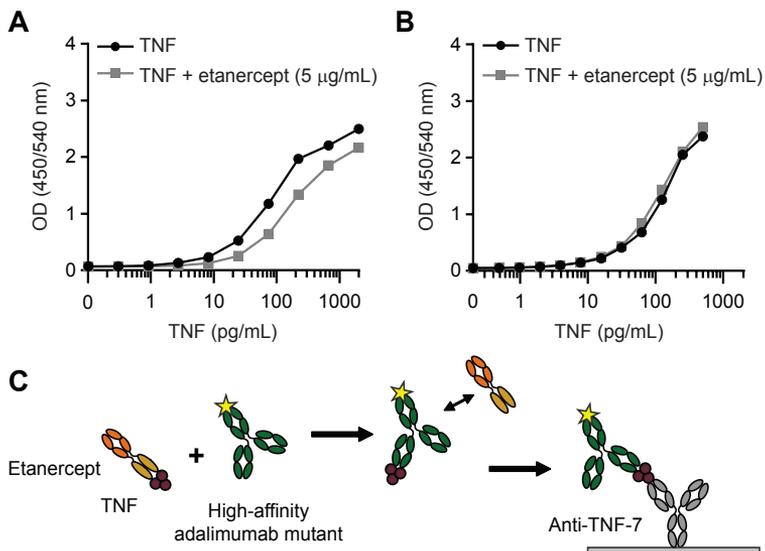
### **Competing interests**

M.T.N. reports having received consultancy fees from Abbott, Roche, Pfizer, Merck Sharp & Dohme (MSD), Union chimique Belge (UCB), Swedish Orphan Biovitrum and Bristol-Myers Squibb (BMS), and payment for lectures from Abbott, Roche and Pfizer. G.J.W. has received a research grant from Pfizer (paid to the institution) and honoraria for lectures from Pfizer, UCB, AbbVie, Biogen and BMS. T.R. has received honoraria for lectures from Pfizer, AbbVie and Regeneron, and a research grant from Genmab. The other authors have declared no conflicts of interest.

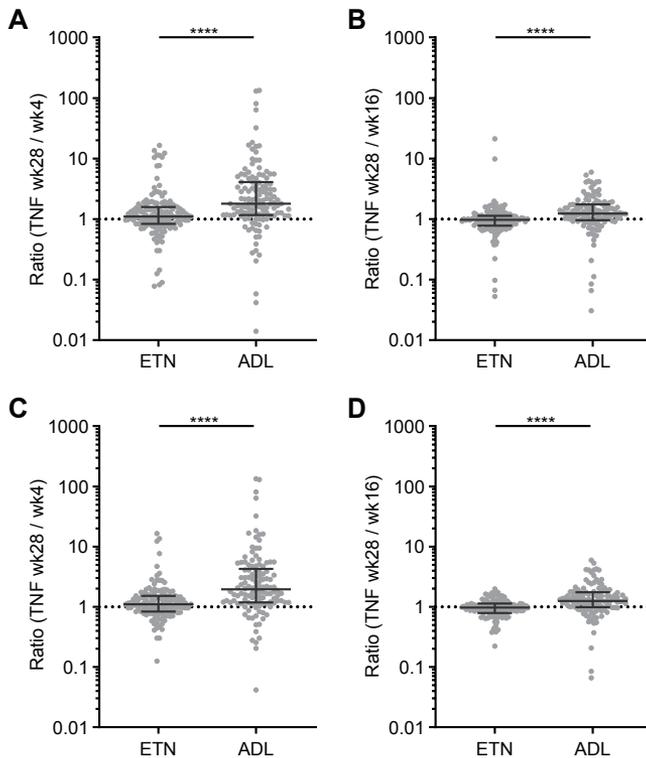
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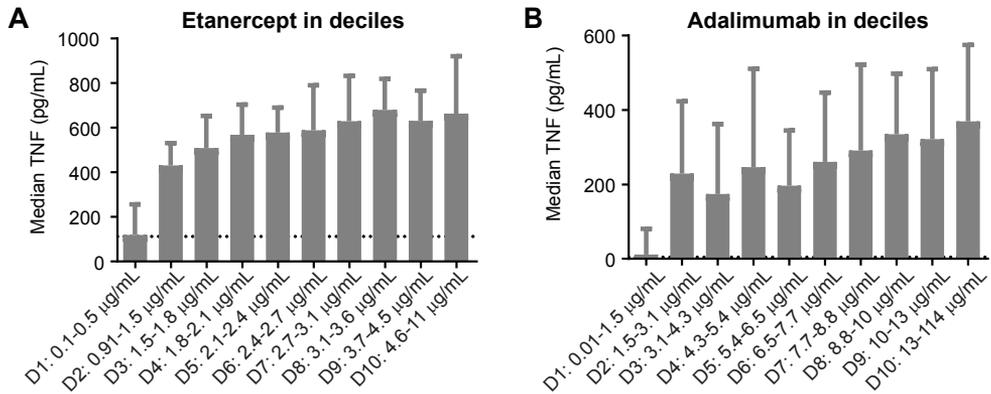
## Supplemental Materials



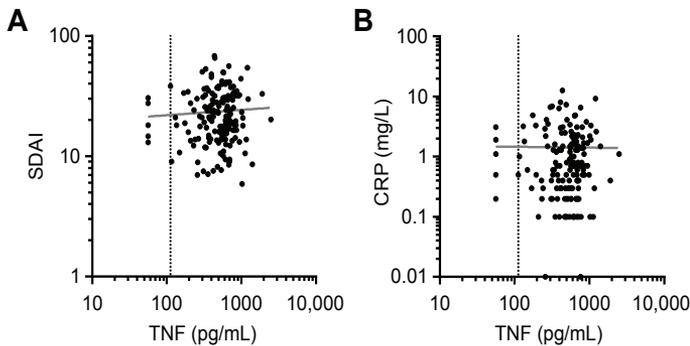
**Supplemental Figure S1. Development of a drug-tolerant competition ELISA.** Quantification of free TNF and TNF-etanercept complexes with a conventional TNF ELISA (**A**) and with the drug-tolerant competition ELISA (**B**). Shown is a representative titration of TNF, preincubated in absence or presence of 5 µg/mL etanercept of at least three independent experiments. (**C**) Schematic overview of the drug-tolerant competition ELISA. An excess of a biotinylated high-affinity adalimumab mutant antibody in fluid phase is added, which will result in the displacement of etanercept from TNF. These TNF-adalimumab mutant antibody complexes are bound to an anti-TNF coating antibody.



**Supplemental Figure S2. Ratio of TNF concentrations.** (A) Ratio of TNF concentrations in patients treated with etanercept (ETN) or adalimumab (ADL) at week 28 over week 4 (median of 1.11 and 1.80, respectively). (B) Ratio of TNF concentrations at week 28 over week 16 (median of 0.97 and 1.24 for etanercept and adalimumab, respectively). (C-D) Results were comparable when samples were only included in the analysis if the drug concentration exceeded 0.1 µg/mL. Median ratio for etanercept was 1.10 and 0.97 in C and D, respectively. Median ratio for adalimumab was 1.95 and 1.26 in C and D, respectively. (A-D) Black lines show median (IQR); dotted lines indicate a ratio of 1, meaning that TNF concentrations were similar at the indicated visits. \*\*\*\* P < 0.0001, Mann-Whitney U test.



**Supplemental Figure S3. Relation between TNF and drug concentrations.** Median (IQR) TNF concentration at week 4 determined in the deciles formed according to etanercept (A) or adalimumab (B) concentrations at week 4. X-axis show the range in etanercept or adalimumab concentrations of each decile. Dotted lines indicate cut-off of TNF (112 and 5 pg/mL in A and B, respectively).



**Supplemental Figure S4. Relation between week 4 TNF concentrations and baseline disease activity.** Correlation between week 4 TNF concentrations and (A) disease activity at baseline according to simplified disease activity index (SDAI) (Spearman's  $\rho = 0.047$ ,  $P = 0.54$  ( $n = 170$ )) or (B) CRP (Spearman's  $\rho = 0.055$ ,  $P = 0.47$  ( $n = 170$ )). Gray lines indicate log-log linear fit, weight by  $1/Y^2$ . Patients without detectable etanercept ( $<0.1$  µg/mL) at week 4 ( $n = 7$ ) were excluded from the analysis. Dotted lines indicate cut-off of TNF (112 pg/mL).

**Supplemental Table S1. Demographics, previous and concomitant therapies, and disease status at baseline.**

	Patients (n = 186)
<i>Demographics</i>	
Age, mean ± SD (years)	52 ± 12
Female, no. (%)	148 (80)
BMI, mean ± SD	25.9 ± 5.6
<i>DMARD therapy</i>	
Prior DMARDs, median (IQR)	3 (2-3)
MTX use, no. (%)	150 (81)
MTX dose, median (IQR) (mg/week)	25 (15-25)
Prednisone use, no. (%)	53 (29)
Prednisone dose, median (IQR) (mg/day)	7.5 (5.0-10.0)
<i>Disease Status</i>	
Disease duration, median (IQR) (years)	5 (2-14)
ACPA positive, no. (%)	129 (69)
IgM-RF positive, no. (%)	128 (69)
Erosive, no. (%)	124 (67)
DAS28, mean ± SD	5.2 ± 1.2
SDAI, mean ± SD	21 (15-31)
TJC28, median (IQR)	7 (4-14)
SJC28, median (IQR)	6 (3-9)
VAS pain, median (IQR) (mm)	64 (49-77)
VAS global, median (IQR) (mm)	62 (49-79)
VAS physician, median (IQR) (mm)	56 (41-66)
ESR, median (IQR) (mm/hour)	20 (10-37)
CRP, median (IQR) (mg/liter)	7 (3-19)
HAQ, median (IQR)	1.25 (0.75-1.72)

SD, standard deviation; no., number; IQR, inter quartile range; BMI, body mass index; DMARD, disease-modifying anti rheumatic drug; MTX, methotrexate; ACPA, anti-citrullinated protein antibody; IgM-RF, IgM rheumatoid factor; DAS28, 28-joint disease activity score; TJC28, tender joint count (28 joints); SJC28, swollen joint count (28 joints); SDAI, simplified disease activity index; ESR, erythrocyte sedimentation rate; CRP, C-reactive protein; HAQ, Health Assessment Questionnaire.