(Anti-)TNF alpha matters in rheumatoid arthritis
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Chapter 2

The clinical response to infliximab in rheumatoid arthritis is in part dependent on pre-treatment tumour necrosis factor alpha expression in the synovium.

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Chapter 2

ABSTRACT

Objective.
To determine whether the heterogeneous clinical response to TNFα blocking therapy in rheumatoid arthritis (RA) can be predicted by TNFα expression in the synovium before initiation of treatment.

Methods.
Prior to initiation of infliximab treatment, arthroscopic synovial tissue biopsies were obtained from 143 patients with active RA. At week 16 clinical response was evaluated using the Disease Activity Score (DAS28). Immunohistochemistry was used to analyze the cell infiltrate as well as the expression of various cytokines, adhesion molecules, and growth factors. Stained sections were evaluated by digital image analysis. T-test were used to compare responders (decrease in DAS28 $\geq$ 1.2) with non-responders (decrease in DAS28 <1.2) and multivariable regression was used to identify the independent predictors of clinical response.

Results.
Synovial tissue analysis confirmed our hypothesis that the baseline level of TNFα expression is a significant predictor of response to TNFα blocking therapy. TNFα expression in the intimal lining layer and synovial sublining were significantly higher in responders than in non-responders ($P = 0.047$ and $P = 0.008$, respectively). The numbers of macrophages, macrophage subsets, and T cells (all able to produce TNFα) were also significantly higher in responders than in non-responders. The expression of IL-1β, IL-6, IL-18, IL-10, E-selectin, ICAM-1, VCAM-1, VEGF, and bFGF was not associated with response to anti-TNFα treatment.

Conclusion.
The effects of TNFα blockade are in part dependent on synovial TNFα expression and infiltration by TNFα producing inflammatory cells. Clinical response cannot be predicted completely, indicating involvement of other as yet unknown mechanisms.
Tumor Necrosis Factor alpha (TNFα) blocking agents as treatment for rheumatoid arthritis (RA) were developed based on evidence that the pro-inflammatory cytokine TNFα plays an important role in the pathogenesis. Some patients however do not clinically respond to TNFα blockade. At present no factors have been identified that fully explain or predict the differential response.

One explanation for the heterogeneous clinical response may be found in the baseline variability in TNFα expression among individual patients. Genetic studies have suggested that individuals predisposed to high TNF production could show worse responses to anti-TNFα therapy. In contrast, a recent study using an in vitro bioassay suggested that good responsiveness to anti-TNF therapy is associated with significantly higher TNFα bioactivity at baseline compared to non-responding patients. Taken together, it remains to be determined which baseline cytokine profile distinguishes responding from non-responding patients in vivo.

Another explanation for the diversity in response may be that inflammatory mediators other than TNFα drive different pathogenetic subsets of RA.

We hypothesized that the pre-treatment TNFα level in the synovium might be related to clinical efficacy, where TNFα blocking therapy could be most effective in patients with high pre-treatment TNFα levels, as previously suggested in a small pilot study. In a prospective study we obtained arthroscopic synovial tissue samples from 143 RA patients prior to initiation of infliximab therapy. We examined the cell infiltrate as well as the expression of cytokines, adhesion molecules, and growth factors to identify predictors of clinical response.

**PATIENTS AND METHODS**

**Patients.**

Consecutive patients with RA according to the ACR criteria were enrolled in the study. All failed at least two disease modifying anti-rheumatic drugs (DMARDs) including methotrexate (MTX), and had a disease activity score (DAS28) ≥ 3.2 when included in the study. Patients were on stable maximal tolerable MTX treatment (5-30 mg/week). Oral corticosteroids (≤ 10 mg/day) and non-steroidal anti-inflammatory drug (NSAIDs) were allowed if stable for at least one month prior to baseline. Concomitant medication was kept stable throughout the study. Previous use of a TNF blocking agent was an exclusion criterion. The Medical Ethics Committee of the Academic Medical Center, University of Amsterdam approved the protocol. All patients gave written informed consent.

**Treatment and evaluation of clinical response.**

All patients were treated with infliximab according to the label for RA in a dosage of 3 mg/kg intravenously at baseline, week 2, 6, and subsequently every 8 weeks. The DAS28 was evaluated at baseline, week 4, 8, 12, and 16 by specially trained research nurses.
For the analysis the absolute change in DAS28 (delta DAS28) at week 16 was dichotomized and defined as non-response (delta DAS28 < 1.2) versus response (delta DAS28 ≥ 1.2). The dichotomy of the delta DAS28 (on average comparable with a 20% improvement in DAS28) was chosen because it is applied in daily clinical practice and required for prolongation of reimbursement for TNFα blocking therapy by insurance companies in the Netherlands. Response was evaluated at 16 weeks because a significant improvement is expected to occur within 3 to 4 months, after which alternative treatment should be considered.\[8\]

**Arthroscopy and synovial biopsy.**

Before the first infliximab infusion patients underwent a mini-arthroscopy under local anesthesia to obtain synovial tissue samples from an actively inflamed knee, ankle, wrist or metacarpophalangeal joint.\[9\] Biopsies were taken with a 2-mm forceps (Storz, Tuttlingen, Germany) from 6 or more sites within the joint to minimize sampling error. Biopsies were immediately snap frozen en bloc in Tissue Tek OCT (Miles, Elkhart, IN) after collection. Sections of 5 μm were cut in a cryostat and mounted on Star Frost adhesive glass slides (Knittelgläser, Germany). Slides were stored at -80°C until immunohistochemical staining.

**Immunohistochemical analysis.**

Synovial sections were stained using the following monoclonal antibodies to analyze the infiltrate: anti-CD55 (67:Serotec, Oxford, UK) to detect fibroblast-like synoviocytes (FLS), anti-CD68 (EBM11: DAKO, Denmark) to detect macrophages, anti-CD3 (SK7, Becton Dickinson (BD), CA) for T cells, anti-CD22 (CLB-B-ly/1,6B11, the Netherlands) for B cells and anti-CD38 (HB7, BD) for plasma cells. CD163 (Ber-MAC3; DAKO) was stained to detect resident tissue macrophages; infiltrating macrophages were evaluated by detection of myeloid related proteins MRP8 (8-5c2, BMA biomedicals, Augst, SW) and MRP14 (S36.48, BMA biomedicals). For the detection of cytokines, adhesion molecules and growth factors we used: anti-human TNFα (52B83; Monosan, Belgium), anti-IL-6 (Nephrology Department LUMC, the Netherlands), anti-IL-10 (23738.111, R&D, UK), anti-IL-18 (2d3b6, MD Biosciences, MN), anti-IL-1β (2D8, immunoKontact, UK), anti-ICAM-1 (MEM111, Sanbio, Belgium), anti-VCAM (1G11B1, Sanbio), anti-E-selectin (BBIG-E4, R&D), anti-VEGF (N.010313 C-1, Santa Cruz, CA), and anti-bFGF (F14220, BD). Staining of cellular markers was performed using a three-step immunoperoxidase method.\[2\] For staining of cytokines, adhesion molecules, and growth factors biotinylated tyramine was used as amplification. For control sections the primary antibody was omitted or irrelevant immunoglobulins were applied.

**Digital image analysis.**

All sections were analyzed at random by trained analysts blinded for clinical outcome. Images were acquired and analyzed by computer-assisted image analysis using a Syndia algorithm on a Qwin-based analysis system (Leica, Cambridge, UK).\[10\] Per marker 18 high-power fields were analyzed. Cellular markers were expressed as positive cells/mm² (counts/mm²). Staining
of cytokines, adhesion molecules, and growth factors was expressed as integrated optical density/mm² (IOD/mm²). CD68+ macrophages and TNFα expression were analyzed separately in the intimal lining layer and the sublining.

Statistical analysis.

Independent Student’s t-tests or Mann Whitney U tests were used to detect significant differences in baseline parameters between responders and non-responders. The Chi-square test was employed to compare the percentage erosive, IgM-RF and anti-CCP positive patients in the responder and non-responder groups. Variables with a P value < 0.1 in the univariable analysis were selected as possible predictors in a stepwise backward multivariable logistic regression model. Several exchangeable prediction sets were constructed based on the correlation structure among the potential predictors with strongly correlating predictors in different sets. The model with the highest explained variance according to Nagelkerke is reported. Correlations were assessed with the Pearson product-moment or Spearman rank-order correlation coefficients, whichever was appropriate. SPSS 11.1.4 (Chicago, IL) was used. Because the primary objective of this study was to find a pathogenetic mechanism in synovial tissue relating to clinical response, a per-protocol analysis was performed.

RESULTS

Enrollment, discontinuation and exclusion. One hundred forty-three patients were enrolled. The population was predominantly female (74%), rheumatoid factor positive (71%) with a mean age of 55 ± 13 (± standard deviation, SD) years, and a mean disease duration of 125 ± 108 months. Erosions were present in 110 (77%) patients. The mean DAS28 score was 5.9 ± 1.0 at baseline and the mean MTX dose was 18.2 ± 8.5 mg/week. In 3 patients a flare of arthritis at week 6 (n=1) and week 12 (n=2) was the reason for discontinuation. These patients were analyzed as non-responders. Patients (n=18) who violated the protocol or discontinued infliximab therapy (for reasons other than non-response) were excluded.

Synovial tissue exclusion. Following strict quality control, 23 synovial biopsies were excluded by a blinded assessor for absence of an intimal lining layer. After excluding 18 patients from the response analysis and 23 synovial biopsies from the tissue analysis a total of 103 completely evaluable patients remained for the analysis of synovial tissue in combination with clinical response.

Baseline patient characteristics.

The remaining group of 103 patients who were analyzed for response in combination with synovial tissue consisted of 71 females and 32 males, of whom 75% were rheumatoid
factor positive. The mean age was 55 ± 13 years, and the mean disease duration was 125 ± 110 months. Erosions were present in 79 (77%) patients. The mean DAS28 score was 5.9 ± 1.1 at baseline and the mean MTX dose was 18.8 ± 8.5 mg/week. Corticosteroids were used by 28 (27%) patients with a mean dose of 8.0 ± 2.9 mg/day. On average, patients had failed treatment with 2.2 DMARDs before inclusion in the study. No notable changes occurred in the baseline characteristics of the analyzed 103 patients compared to the baseline characteristics of all 143 enrolled patients (see Table 1).

**Clinical response.**

Seventy of 103 patients (68%) were DAS28 responder and 33 (32%) non-responder at week 16. The clinical efficacy matched the expected 60-70% responders observed in previous randomized controlled trials with infliximab in RA patients. The mean change in DAS28 was 1.84 ± 1.26 at week 16. For non-responders the average delta DAS28 was 0.45 ± 0.58 compared to 2.52 ± 0.89 for responders. This difference was highly significant (P < 0.001).

All baseline patient characteristics were also tested for differences between responders and non-responders. The DAS28 at baseline was nearly significant and was included in the prediction model (P = 0.07). The same holds for anti-CCP positivity (P = 0.08). Finally, there were significantly more rheumatoid factor positive patients in the responder compared to the non-responder group (P = 0.020), (see Table 1).

<table>
<thead>
<tr>
<th>Table 1. Baseline patient characteristics.</th>
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<td><strong>Demographics</strong></td>
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<td><strong>Disease status</strong></td>
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<td><strong>Disease duration (months)</strong></td>
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<td><strong>Erosive disease (%)</strong></td>
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* Mean values ± standard deviation (SD), median and interquartile range (IQR) or percentages are shown. P values < 0.05 (two-sided) are significant and shown in *Italic*.
Baseline synovial TNFα expression and the number of major TNFα producing cells are associated with clinical response (Δ DAS28 ≥ 1.2) after 16 weeks of TNFα blocking therapy.

Scatter diagrams of the synovial predictors in individual patients in both response groups are presented in Figure 1. TNFα expression levels were higher in the synovial sublining of responders compared to non-responders (P = 0.008) (Figure 1 A). Similarly, TNFα expression in the

![Figure 1 A](image)

Figure 1. A) The median synovial sublining TNFα expression was higher in responders compared to non-responders (P = 0.008). B) The median number of CD3+ T cells in responders versus non-responders (P = 0.001). C) The mean number of CD68+ sublining macrophages was also higher in responders 576 ± 428, than in non-responders 387 ± 338 (P = 0.029). D) CD163+ macrophages in responders versus non-responders (P = 0.017).
intimal lining layer was higher in responders compared to non-responders (P = 0.047). Table 2 shows the associations of synovial markers and response.

Because macrophages are known to be the main TNFα producing cells in the synovium, the number of macrophages was studied. The mean number of CD68+ sublining macrophages was significantly higher in responders than in non-responders (P = 0.029) (Figure 1 C). Similarly, the number of CD163+ resident tissue macrophages as well as the number of infiltrating MRP8+ and MRP14+ macrophages was higher in responders (P = 0.017, P = 0.018 and P = 0.024, respectively) (Figure 1 D). Finally, the median number of CD3+ T cells was higher in responders (P = 0.001) (Figure 1 B).

Baseline TNFα is the only significant synovial predictor of response.

The relationship between TNFα expression and clinical response was confirmed by stepwise backward multivariable logistic regression analysis. Due to multicollinearity among predictors ten exchangeable bivariate prediction sets were constructed consisting of 1) TNFα expression

| Table 2. Associations of studied synovial predictors with clinical response. |
|-----------------------------|-----------------------------|-----------------------------|
|                            | Responders | Non-responders | P value |
|                            | N= 70      | N= 33          |         |
|                             | Delta DAS28 score |                       |
| Cytokines (IOD/mm²)         | ≥ 1.2      | <              |
| TNFα lining                 | 48214 (29116-88971) | 36376 (23394-54271) | 0.047 |
| TNFα sublining              | 77947 (38710-123535) | 46033 (19890-78129) | 0.008 |
| IL-1β                       | 56278 (31063-81655) | 54484 (32098-129041) | 0.814 |
| IL-6                        | 50537 ± 41939 | 38364 ± 28527 | 0.135 |
| IL-10                       | 131106 ± 87359 | 115964 ± 83953 | 0.415 |
| IL-18                       | 9530 ± 12086 | 7009 ± 7470 | 0.289 |
| Cellular markers (counts/mm²)|            |                |         |
| CD55                        | 652 ± 418 | 694 ± 389 | 0.895 |
| CD3                         | 149 (66-385) | 47 (22-163) | 0.001 |
| CD68 lining                 | 374 ± 253 | 295 ± 184 | 0.130 |
| CD68 sublining              | 576 ± 428 | 387 ± 338 | 0.029 |
| CD163                       | 1100 ± 432 | 878 ± 433 | 0.017 |
| CD22                        | 54 ± 99 | 47 ± 111 | 0.756 |
| CD38                        | 284 ± 384 | 325 ± 556 | 0.661 |
| MRP8                        | 139 (35-294) | 53 (19-135) | 0.018 |
| MRP14                       | 159 (34-526) | 51 (18-166) | 0.024 |
| Adhesion molecules (IOD/mm²)|            |                |         |
| ICAM                        | 30790 ± 33016 | 22321 ± 21706 | 0.195 |
| VCAM                        | 80065 ± 52630 | 70701 ± 44574 | 0.387 |
| E-Selectin                  | 37651 ± 40134 | 30488 ± 29972 | 0.373 |
| Growth factors (IOD/mm²)    |            |                |         |
| VEGF                        | 18252 ± 78689 | 5776 ± 5372 | 0.374 |
| BFGF                        | 209 ± 736 | 64 ± 163 | 0.276 |

* Data are presented as mean ± SD or median (interquartile range) whichever appropriate. P values < 0.05 (two-sided) are significant and shown in Italic.
1. in either the intimal lining layer or synovial sublining with 2) one out of five cellular markers.

2. This revealed that TNFα expression in the synovial sublining was the only independent, early
determinant of therapy response (P = 0.011), explaining just about 10% of the variance in
response to therapy (R² = 0.099, according to Nagelkerke, odds ratio (OR) = 1.013). Likewise,
TNFα expression in the intimal lining layer rather than synovial sublining explained 9% of the
variance in response to therapy (P = 0.020), (R² = 0.089, OR = 1.017). Thus, after adjustment for
TNFα in a bivariate logistic regression model including either the numbers of CD68+ sublining
macrophages, CD163+, MPR8+, MRP14+ macrophages, or CD3+ T cells, these cellular markers
were no longer significantly associated with response. The removal of these cellular markers
from the model is consistent with the notion that these cells are the main producers of TNFα.

Contribution of disease activity at baseline to the prediction of response.

3. To assess the value of a combined prediction model consisting of both clinical and synovial
data we added the relevant clinical variables (DAS28 score, the presence of IgM-RF or anti-CCP
antibodies) to the 10 bivariate synovial models. With stepwise backward multivariate logistic
regression this resulted in a significant prediction model with TNFα expression in the synovial
sublining (P = 0.008, OR = 1.014) and the DAS28 (P = 0.031, OR = 1.611) with an increased
explained variance of 17% (R² = 0.172). A similar analysis except with TNFα expression in the
intimal lining layer (P = 0.023, OR = 1.017) resulted in a significant model including the DAS28 (P
= 0.044, OR = 1.588) and the presence of anti-CCP antibodies (P = 0.046, OR = 3.038) (R² = 0.182).

Baseline IL-1β, IL-6, IL-18, and IL-10 expression is not associated with response to TNFα blocking therapy.

4. In addition to TNFα, we studied the expression of other cytokines such as IL-18 which is
believed to operate mainly upstream of TNFα, while IL-1β and IL-6 on the other hand appear
to act mainly downstream of TNFα. Despite the known interplay of these cytokines with TNFα
we found no relationship between synovial expression levels of either of these cytokines and
clinical response. We did find, however, a significant correlation between the level of IL-1β
expression and TNFα expression (r = 0.306, P = 0.014). The expression level of IL-10 was not
different between response groups (see Table 2).

Baseline expression of adhesion molecules and angiogenic factors is not associated with response to TNFα blocking
therapy.

5. In accordance with their function in leukocyte recruitment a significant correlation was found
between the number of sublining macrophages on the one hand and the expression of the
vascular adhesion molecules E-selectin (Spearman’s rho correlation coefficient 0.275-0.536, P
< 0.01) on the other. However, the level at which the adhesion molecules were expressed in
the synovium at baseline did not differ between responders and non-responders. Furthermore,
VEGF is known to mediate angiogenesis and is regulated by pro-inflammatory cytokines like
TNFα. However, no association was found between VEGF expression and response (Table 2). Similarly, no difference was found in the expression of bFGF.

**DISCUSSION**

The primary objective of this study was to investigate whether immunohistologic assessment of the cell infiltrate and cytokine expression in the synovium prior to initiation of TNFα blocking therapy could predict clinical response in patients with RA. We could confirm our hypothesis that the level of synovial TNFα expression is a significant early predictor of the response. This was shown by increased TNFα expression levels in both the intimal lining layer and synovial sublining of responding compared to non-responding patients. In line with these findings, there was increased infiltration by macrophages, including both CD163+ resident tissue macrophages and MRP8+ and MRP14+ infiltrating macrophages, as well as T cells in responders versus non-responders. It is important to note that these cells are the main source of TNFα in the synovium of RA patients.[11]

Consistent with the clinical experience that the response to TNFα blockade is not a dichotomous phenomenon, [12] there was no distinct threshold value in TNFα expression in the synovium of RA patients. Multivariate logistic regression analysis of synovial markers showed that TNFα expression in the sublining could explain about 10% of the variance in response to therapy. After adjusting for disease activity at baseline this further increased to 17%. Hence, the predictive value of synovial TNF expression is statistically significant, but overall limited. This clearly indicates that variables other than synovial TNFα expression are involved as well. The results presented here show for the first time that the expression of IL-1β, IL-6, IL-18, IL-10, E-selectin, ICAM-1, VCAM-1, VEGF, and bFGF is not associated with clinical response to anti-TNFα treatment. Thus, the features of synovial inflammation at baseline in responders to anti-TNF therapy compared to non-responders do not merely represent a greater amount of inflammation, but the results presented here underscore the importance of specific inflammatory pathways. Future work should

The search for predictors of response is important in the context of personalized medicine, which may be an effective approach to increase the percentage of patients exhibiting a robust response to a given treatment. Previous work has clearly suggested that RA consists of different pathogenetic subsets, leading to common signs and symptoms associated with what we at present define clinically as RA.[2, 3, 13] Thus, it is conceivable that for instance TNFα expression is more important in some patients than in others, and that TNF blockade could be more effective in the former. Although we are as yet not able to predict the response sufficiently to select patients who are likely to have a beneficial response to a specific treatment before
1. initiation of therapy and to guide treatment decisions, the results of the present study do pro-
2. vide proof of principle that this might be achieved by further optimization of the biomarkers
3. or perhaps combinations with other clinical and biological variables that need to be identified.
4. The relevance of this approach is underscored by the expanding array of biological therapies
5. and their costs. [14]
6. 
7. There is no evidence that simple measurement of plasma TNFα levels can be used to predict
8. clinical response to TNFα blockade. As it appears logical that patients producing high levels
9. of TNFα at the site of inflammation are more likely to benefit from TNFα blockade than those
10. with lower TNFα levels, we focused on the synovium as the primary target of RA. The associa-
11. tion between pre-treatment TNFα expression in the synovium and clinical response to 3 mg/
12. kg of infliximab described here in 103 RA patients confirms and extends a trend observed in
13. a pilot study in RA patients where 4 out of 8 patients who met the ACR50 response criteria at
14. 2 weeks after initiation of infliximab (10 mg/kg) therapy were those with the highest levels of
15. TNFα expression in the synovium, as shown by immunohistochemistry.[7] Of interest, the find-
16. ings are also in accordance with a recent study suggesting that clinical response at 52 weeks
17. is associated with significantly higher circulating TNFα bioactivity (measured by an in vitro
18. bioassay) at baseline in responding compared to non-responding patients.[6] Together, these
19. studies support the notion that the initial clinical response to TNFα blockade is related to pre-
20. treatment levels of TNFα production. In patients who initially respond, but loose response over
21. time, other mechanisms like formation of antibodies against the drug may be operative.[15]
22. 
23. In conclusion, the results presented here show proof of principle that the heterogeneous
24. response to TNFα blockade is associated with TNFα expression in the inflamed synovium.
25. Future work should expand the search for other biomarkers and molecular networks as well as
26. combinations with clinical variables. Thus, the prediction of how a patient will respond might
27. come in reach of the treating physician.
28. 
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