Immuno-monitoring reveals an extended subclinical disease activity in tocilizumab-treated giant cell arteritis

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Abstract

Objective. Tocilizumab is effective in inducing and maintaining remission of GCA. Despite clinical and serological control of disease, magnetic resonance angiography may show persistence of inflammatory signals of unknown significance in arterial walls. Thus, there is an unmet need for tools to detect subclinical disease activity.

Methods. Immune-inflammatory markers were measured in prospectively collected sera of the first randomized, double-blind, placebo-controlled trial investigating the use of tocilizumab in GCA. As a comparison, immune-inflammatory markers were also measured in sera from age- and sex-matched healthy volunteers. The biomarkers were quantified using luminex technology.

Results. Of all the parameters determined, only MMP-3, pentraxin-3 and sTNFR2 were significantly elevated, while ICAM-1 and CD163 were significantly decreased during the early stages of the study, at time points of full clinical remission under treatment with tocilizumab plus glucocorticoids. In contrast, tocilizumab monotherapy towards the end of the study resulted in an almost complete normalization of immune-inflammatory molecules, as defined by the healthy controls. MMP-3 levels showed a weak association with magnetic resonance signal intensity; none of the biomarkers predicted relapse occurring within 6 months after study end.

Conclusion. The data documented a subclinical disease activity in GCA that was more pronounced during the early stages of treatment and almost disappeared towards the study end. They indicated that tocilizumab treatment of at least 52 weeks is necessary in order to reset a broad range of immune-inflammatory pathways.


Key words: giant cell arteritis, tocilizumab, glucocorticoids, biomarkers, disease activity, MMP-3

Introduction

GCA is the most frequent large vessel vasculitis in Western countries, affecting predominantly women over 50 years of age [1, 2]. It is histologically characterized by intense inflammation in all layers of the medium- to large-sized arteries and the formation of granulomas. Risks are obliteration of the vascular lumen, resulting in blindness, and aneurysm formation, with rupture of the aorta. Conventional treatment consists of high-dose and long-term glucocorticoids (GCs), leading to side effects over time. MTX helps to reduce the risk of relapse and to spare GCs [3]. Tocilizumab (TCZ) has very recently been proved to maintain remission and to lead to a highly significant reduction of the cumulative GC dose [4, 5].

Between 2012 and 2015, we performed the first phase 2, randomized, double-blind, placebo-controlled trial (RCT) to define the role of TCZ in inducing and controlling remission of GCA (ClinicalTrials.gov registration number: NCT01450137) [4]. Whereas all but one patient in the TCZ arm stayed in lasting remission up to study end at week 52, only two patients in the placebo arm did not relapse.
Patients with initial positive MR-angiography (MRA findings) underwent control MRA at weeks 12 and 52. At week 12, three out of nine TCZ patients (33%) showed normalization of vessel wall signals. At week 52, there was additional MRA improvement in some TCZ patients, but one-third showed persistent or increased late vessel wall enhancement despite clinical remission [6]. The significance of these inflammatory signals is currently unknown.

The protocol of the RCT included sampling of sera at the beginning and prior to every 4-weekly infusion, offering a unique opportunity to study a broad range of immune-inflammatory biomarkers prospectively in a very well characterized patient population. This was of particular interest because remission was initially induced by a combination of GCs plus TCZ and maintained towards study end by TCZ in monotherapy.

The aims of this study were: to search for and characterize a potential subclinical disease activity; to compare the biomarker profiles in early full remission under combined therapy with TCZ plus GCs, and at late stage under TCZ monotherapy; to search for markers associated with persistent MRA signals in vessel walls; and to search for markers predicting relapse within 6 months after study end.

Methods

Patients and controls

Thirty patients aged 50 years or older suffering new-onset or relapsing GCA (confirmed by either temporal artery biopsy or by MRA, and meeting the 1990 ACR criteria) were included in the RCT [4]. Of these, 20 received TCZ, which was dosed as for treating RA, that is, 8 mg/kg bodyweight i.v. in 4-weekly intervals. GC co-medication was started at a dose of 1 mg/kg bodyweight and rapidly reduced thereafter: −0.1 mg/kg bodyweight weekly until week 8, then −0.05 mg/kg bodyweight weekly until week 12, followed by monthly −1 mg/day. This reduction scheme resulted in a mean daily dose of 7 mg prednisone at week 12 and a discontinuation of GCs between months 9 and 11. A prior treatment with prednisolone up to 1 mg/kg bodyweight for a maximum of 10 days between inclusion in the trial and the first infusion was permitted.

Blood samples were collected at weeks 0, 2, 4, 6, 8, 10 and 12 and in 4-weekly intervals thereafter until week 52. The serum samples were immediately processed and stored in aliquots of 0.5 ml at −70 °C.

Sera of TCZ patients with relapse (n = 1), serious adverse events (n = 2) or with incomplete sampling (n = 3) were excluded from analysis. Therefore, the longitudinally analysed cohort comprised 14 TCZ patients in lasting remission for 52 weeks. Sera of patients in the placebo arm were purposely not analysed because GC treatment had to be re-increased repeatedly, resulting in a very inhomogeneous patient population.

For every patient, a serum of an age- and sex-matched healthy control was collected. These healthy controls lived on their own, did not take any regular medication or anti-inflammatory agent, and did not show signs of infection within 4 weeks of venipuncture.

Biomarkers and methods

Based on recent studies and pathophysiological considerations, a broad range of biomarkers was chosen (supplementary Table S1, available at Rheumatology online). They included proteins of the TNF superfamily and the IFN family, T lymphocytes and regulatory T cells, monocytes, B lymphocytes, inflammatory markers such as high sensitivity CRP, adipocytokines, and catabolic enzymes such as MMPs. Investigations were performed with luminex technology (R&D Systems, Minneapolis, USA and Invitrogen, Carlsbad, USA). Samples were quantified on a multiplex system [Bio-Plex 100 array reader with Bio-Plex Manager software (version 6.1); Bio-Rad, Hercules, CA, USA]. If not specified, protein concentrations are given in pg/ml. In addition, TCZ, IL-6 and sIL-6 concentrations were determined by an external company using luminex technology (QPS, Groningen, the Netherlands).

MRA and relapses

Magnetic resonance signals were analysed as described elsewhere [8]. In brief, MRA was repeated at 3 months and at study end if patients showed signs of mural inflammation at recruitment. Vessel wall signals were judged by two experienced radiologists in blinded fashion using the following grading: 0 = no mural thickening (maximal vessel wall thickness <2.3 mm), no enhancement; 1 = no thickening, slight mural enhancement; 2 = mural thickening (>2.3 mm), significant mural enhancement; 3 = strong thickening (>3 mm), strong mural and perivascular enhancement. Correlations were calculated between biomarkers and 21 MRA signals [for 7 patients with MRA examinations at all three time points).

Statistical analysis

Statistical analysis was performed using GraphPad Prism 5.0 software and IBM SPSS Statistics 21. Welch’s correction was used to calculate the significance of differences between paired sample groups, and the Mann-Whitney U test was used to calculate the significance of differences between unpaired sample groups. Correlation analysis was done by calculation of Spearman rank’s correlation coefficient. To search for biomarkers predicting flare and mural signal intensity, binary logistic regression and univariate receiver operating characteristic curves were generated.

Ethical approval and patient informed consent

The amendment to study 168/10 (ClinicalTrials.gov registration number: NCT01450137) was approved by the ethical commission of Bern, Switzerland 4 May 2016, and the study was done in accordance with the Declaration of Helsinki. All patients gave written informed consent.

Results

TCZ, IL-6 and sIL-6R

TCZ levels approached steady-state levels by the time of the sixth or seventh dose (Fig. 1A). Mean pre-dose levels of IL-6 initially increased in response to treatment with TCZ, and subsequently decreased to 50.83 (43.08) by
week 52 (Fig. 1B). Levels of sIL-6R increased almost immediately after initiation of dosing and appeared to reach a plateau after the fifth or sixth dose (Fig. 1C).

Biomarkers not detectable in sera or without dynamic changes over time
Eighteen molecules were not present at detectable levels (supplementary Table S1, available at Rheumatology online). Remarkably, T cell cytokines such as IL-2, IL17A and IFN-γ were neither detectable prior to the first TCZ infusion nor at study end.

Several of the measured molecules showed a tendency to fluctuate but did not display a significant increase or decrease over time (supplementary Table S1, available at Rheumatology online). Values of sTNFR2 and ICAM-1 did not show dynamic changes but differed compared with healthy controls (Fig. 2B). Osteopontin levels were slightly higher at study start, and they became indistinguishable from controls at study end (P = 0.44; data not shown).

Biomarkers with significant changes over time
Pentraxin-3 and MMP-3 serum levels showed a remarkable decrease, while CD163 exhibited a continuous increase over time, reaching almost normal values at the end of the study (Fig. 2A). Pentraxin-3 started at a median concentration of 6342 (95% CI: 3965, 8720) and decreased to 2807 (95% CI: 1993, 3621; P = 0.0002). MMP-3 decreased from a median concentration of 76370 (95% CI: 65721, 87018) to 27642 (95% CI: 21207, 34077; P = 0.0001). MMP-3 levels showed a very homogeneous pattern with little inter-individual variation. At week 4, MMP-3 values were indistinguishable from the values before the first TCZ infusion; thereafter, a steady decrease could be noted, reaching values close to normal. On the other hand, CD163 started at a median concentration of 892198 (95% CI: 628448, 1155947), increased over time and equaled levels of healthy controls at the end of the study, with a median concentration of 1342169 (95% CI: 931206, 1753133; P = 0.0012).

Comparison between sera concentrations at study end and matched healthy controls
Despite complete remission of disease, MMP-3, pentraxin-3 and sTNFR2 levels remained elevated, while ICAM-1 concentrations remained below levels of healthy matched controls (Fig. 2). At the end of the study, the median serum concentration of MMP-3 (27642; 95% CI: 21207, 34077) remained higher compared with that of controls (19400; 95% CI: 14622, 24178; P = 0.0353). The same applied to pentraxin-3 (2807; 95% CI: 1993, 3621) compared with controls (1491; 95% CI: 877, 2105; P = 0.0052) and to sTNFR2 (267; 95% CI: 212, 322) compared with controls (147; 95% CI: 120, 174; P = 0.0023). The median serum concentration of ICAM-1 (1497851; 95% CI: 1.11 × 10⁶ to 1.89 × 10⁶), on the other hand, stayed below the median serum concentration of controls (2246971; 1.83 × 10⁶ to 2.66 × 10⁶; P = 0.0134).

Correlation between biomarkers and MRA signals
An association was found between MMP-3 concentration and the MRA signal intensity of the aortic wall, as determined by MRA, but it did not quite reach the level of significance (R²adj = 0.13492, P = 0.0566; supplementary Fig. S1, available at Rheumatology online).
area under the curve (AUC) did not indicate a significant result (0.821; 95% CI: 0.616, 1.000; $P = 0.093$). A serum concentration of MMP-3 $\geq$ 48,843 was associated with a high mural signal intensity (sensitivity of 77% and specificity of 100%). Values equal or higher showed an effect size of $f^2 = 0.506$, reflecting a strong effect of MMP-3 on mural signal intensity. But the chance to have a high mural signal intensity was not significantly higher with serum concentration $\geq$ 48,843.

Biomarkers associated with relapse after study end
Patients relapsing within 6 months of study end are marked in red and with arrows in Fig. 2. Lower serum concentrations of IL-6 and higher serum concentrations of pentraxin-3 were associated with a higher risk of a flare after study end. The AUC for IL-6 reached 0.764 (95% CI: 0.523, 1.000; $P = 0.068$). A serum concentration of IL-6 of $\leq$ 29.45 was slightly associated with flare after study end (sensitivity of 87.5% and specificity of 55.6%).
The relative risk to flare after study end was 4.44 times higher for serum concentration under 29.45. For pentraxin-3 the AUC reached 0.646 (95% CI: 0.372, 0.920; \( P = 0.312 \)). A threshold of 2241 showed a sensitivity of 66.7% and a specificity of 62.5%. The relative risk to flare after study end was 1.88 times higher for a serum concentration over 2241.

**Correlation of different biomarker and TCZ serum levels**

Table 1 shows correlations between six biomarkers and TCZ serum levels. IL-6 concentrations directly correlate with TCZ levels (\( r = 0.5585, \ P < 0.0001 \), while MMP-3 and pentraxin-3 correlates inversely with TCZ serum level (MMP-3: \( r = -0.5806, P < 0.0001 \), and pentraxin-3: \( r = -0.3855, P = 0.0044 \), respectively).

Several direct and inverse correlations between the six biomarkers could be observed: MMP-3 and pentraxin-3 showed an inverse correlation with IL-6 (\( r = -0.6119; P < 0.0001 \) and \( r = -0.4158; P = 0.0033 \), respectively). MMP-3 and pentraxin-3 serum levels were directly correlated with each other (\( r = 0.4637, P = 0.0005 \)). sTNFR2 also correlated inversely with IL-6 (\( r = -0.3748; P = 0.0087 \), while CD163 demonstrated a positive correlation with IL-6 (\( r = 0.3302; P = 0.0208 \)). In agreement with the positive correlation between CD163 and IL-6, pentraxin-3 and CD163 showed an inverse correlation with each other (\( r = -0.0.2826; P = 0.0403 \)). ICAM-1 was the only biomarker which showed neither a correlation to TCZ serum level nor to the serum level of other biomarkers.

**Discussion**

Two recent RCTs showed effectiveness of TCZ in controlling GCA [4, 5]. Both documented a highly significant and clinically relevant sparing of GCs. Despite these convincing data, questions beyond the clinical outcome remain. In particular, MRA documented the persistence of late enhancement of the arterial walls of several patients [6]. It is currently unknown whether these signals reflect arterial wall inflammation or rather represent persistent hyperaemia only. The quantification of a wide range of immune inflammatory markers may shed light on potential subclinical disease, explain MRA signals and predict relapse after study end.

Due to the fact that all but two patients in the placebo arm suffered relapse(s), the GC dosages had to be re-increased repeatedly, resulting in a highly heterogeneous population. As a consequence, the placebo arm could not serve as a meaningful control. Thus, the presented data characterize the longitudinal evolution of subclinical biological activity only. Whether GC monotherapy would have resulted in comparable changes is not known.

The rise in IL-6 levels after the first dose is typically seen after administration of TCZ and is hypothesized to occur due to displacement of IL-6 as TCZ binds to the soluble and membrane-bound IL-6 receptors [7]. The slow increase of TCZ trough levels implies a delayed control of the disease by the biologic agent and suggests a central therapeutic role of GC in the early stages of the study. Remarkably, the only relapse in the TCZ arm happened in week 11 at a time when GCs were substantially reduced and TCZ had not yet reached steady-state trough levels. New treatment protocols should consider these findings.

Soluble TNFR2 has been proposed as marker of subclinical disease activity in a variety of autoimmune diseases [8-10]. In GCA, however, its performance has not been analysed. In this study, sTNFR2 levels fluctuated over time, and they did not predict relapse. The inter-individual differences are in line with the literature; however, the intra-individual changes remain unexplained. Pentraxin-3 levels have been shown to be associated with vascular inflammation in GCA, identifying patients with very recent optic nerve ischaemia or recent diagnosis [11]. The values of our study show a steady decrease over time, with near normalization at study end. ICAM-1 concentrations, on the other hand, remained below the levels of healthy controls throughout the study. An earlier study documented a rapid fall in ICAM-1 concentration, upon GC treatment, that persisted over the whole study period and reflected clinical remission [12]. The low ICAM-1 levels at the start of our study may be explained by the fact that patients were allowed to receive GC treatment for a maximum of 10 days between inclusion in the trial and the first TCZ infusion. Findings of a more recent trial about infliximab use in the treatment of GCA showed increased levels of ICAM-1 near relapse [13]. As our

### Table 1 Spearman’s rank correlation between different biomarker and TCZ serum levels

<table>
<thead>
<tr>
<th>Biomarker/TCZ</th>
<th>ICAM-1, ( P )-value</th>
<th>sTNFR2 ( P )-value/( r )-value</th>
<th>CD163 ( P )-value/( r )-value</th>
<th>pentraxin-3 ( P )-value/( r )-value</th>
<th>MMP-3 ( P )-value/( r )-value</th>
<th>IL-6 ( P )-value/( r )-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tocilizumab serum level</td>
<td>0.1503</td>
<td>0.8221</td>
<td>0.0945</td>
<td>0.0044/( r ) &lt;-0.25</td>
<td>-0.0001/( r ) &lt;-0.5</td>
<td>-0.0001/( r ) &gt;0.5</td>
</tr>
<tr>
<td>IL-6</td>
<td>0.7020</td>
<td>0.0087/( r ) &lt;-0.25</td>
<td>0.0208/( r ) &gt;0.25</td>
<td>0.0033/( r ) &lt;-0.25</td>
<td>-0.0001/( r ) &lt;-0.5</td>
<td>-0.0001/( r ) &gt;0.5</td>
</tr>
<tr>
<td>MMP-3</td>
<td>0.6078</td>
<td>0.1253</td>
<td>0.1593</td>
<td>0.0005/( r ) &gt;0.25</td>
<td>0.0005/( r ) &gt;0.25</td>
<td>0.0005/( r ) &gt;0.25</td>
</tr>
<tr>
<td>Pentraxin-3</td>
<td>0.4169</td>
<td>0.1784</td>
<td>0.0403/( r ) &lt;-0.25</td>
<td>0.0005/( r ) &gt;0.25</td>
<td>0.0005/( r ) &gt;0.25</td>
<td>0.0005/( r ) &gt;0.25</td>
</tr>
<tr>
<td>CD163</td>
<td>0.1499</td>
<td>0.1395</td>
<td>0.0403/( r ) &lt;-0.25</td>
<td>0.0005/( r ) &gt;0.25</td>
<td>0.0005/( r ) &gt;0.25</td>
<td>0.0005/( r ) &gt;0.25</td>
</tr>
<tr>
<td>sTNFR2</td>
<td>0.5608</td>
<td>0.5808</td>
<td>0.6119</td>
<td>0.6119</td>
<td>0.6119</td>
<td>0.6119</td>
</tr>
</tbody>
</table>

* \( r \) = Spearman’s rank coefficient; positive correlation if \( r > 0 \) and negative correlation if \( r < 0 \).

Bold values mark the significant negative/positive associations.
patients did not show relapses, our findings are in good agreement with these data, and they suggest a sufficient immunosuppression by TCZ monotherapy. CD163 has recently been shown to reflect disease activity in a variety of auto-inflammatory diseases, for example, RA [14]. The subnormal values at early time points in our study may be understood as a profound anti-inflammatory effect of combined GC and TCZ, whereas the gradual increase to normal values under TCZ monotherapy suggests reconstituting homeostasis. A very recent study reports about the use of serum osteopontin as a biomarker of disease activity in GCA and a potential predictor of relapse [15]. Remarkably, our findings show an identical range of initial values and comparable values in remission; however, statistical significance was not reached. Collectively, the data of these biomarkers document an ongoing subclinical disease activity, with quantitative and qualitative changes over time.

Neutralization of IL-6 blunts the acute phase response. The fact that control of the IL-6 pathway using TCZ is sufficient to induce remission in immunologically complex diseases such as RA strongly argues for additional indirect effects, for example, on the recruitment of pathogenic Th cells. Indeed, a study has documented that TCZ abrogates the generation of Th17 cells in RA [16]. In GCA, Th1 and Th17 cells (as well as the CD161+CD4+ precursor cells) have been shown to be massively increased in the arterial wall, whereas Treg cells are reduced locally and in the blood [17]. GC treatment suppresses the Th17 but not the Th1 arm in the blood and the vascular lesions [18]. Very recent data showed that TCZ normalizes deranged function of Treg cells [19]. The fact that we could detect neither IL-17 nor any Th1 cytokines in sera is in line with these findings.

Metalloproteinases play an important role in vessel wall inflammation and destruction of elastic fibres [20, 21]. IL-6 has been shown to induce production of the tissue inhibitor of metalloproteinases [22, 23], and GCs have been shown to induce MMP-3 [24]. These findings imply that neutralization of IL-6, as well as treatment with GC, may lead to uncontrolled activity of catabolic enzymes and propagation of vessel wall destruction, and—in addition—they may serve as an explanation for the known risk of perforation of diverticulitis under dual immunosuppression in GCA. In this regard, the measured levels of metalloproteinase 3 are of particular interest. As displayed in Fig. 2, the values show a uniform pattern over time, with little inter-individual variation. Furthermore, their values decrease significantly towards the study end. Most intriguingly, the MMP-3 values in week 4 are indistinguishable from the values prior to the first infusion of TCZ, although all patients were in full remission at this time point. The values at week 4, therefore, reflect the combined effect of TCZ plus 0.6 mg/kg bodyweight of prednisone, whereas at week 52 patients are on TCZ monotherapy. Taken together, these data argue that targeted inhibition of the IL-6 pathway is sufficient for controlling destructive effector mechanisms. The contradictory results from cell studies and our clinical trial can be reconciled by the hypothesis of indirect effects of TCZ, which eventually overrule the direct effects of IL-6 on tissue inhibitors of metalloproteinases expression.

The calculated correlations between sera concentrations of sTNFR2, ICAM-1, pentraxin-3, CD163, MMP-3 and MRA signals revealed an interesting association between MMP-3 levels and mural signal intensity. Higher levels of pentraxin-3 and lower levels of IL-6 showed a weak correlation with relapse within 6 months after study end. Collectively, however, the data regarding MRA signals and relapse do not suggest that one biomarker or a set of analysed biomarkers will emerge as a clinical tool for guiding treatment.

The main weakness of this study is the small sample size and the lack of a control population. This is partially counterbalanced by the fact that the patients were characterized in great detail, and sera were collected in the context of an RCT.

In summary, our study documents an extended and persistent subclinical disease activity in GCA, which is more pronounced in the early stages of treatment and almost disappears towards the study end. Inhibition of the IL-6 pathway not only abolished signs and symptoms of the systemic inflammation, but prolonged treatment with TCZ appeared to reset a range of inflammatory mechanisms. The data are in line with recent findings about effects of TCZ on Th-17 and Treg generation and function.

Acknowledgements

We thank Diana Dan and Felix Wermelinger for patient care and processing of sera samples, Sandra Gsponer for laboratory assistance and Frauke Feger and Lukas Bütkofer, statistician, of the Clinical Trial Unit of the University of Bern, for advice and supervision of statistical calculations.

Funding: The study was funded by the Research Funds of the Department of Rheumatology and Clinical Immunology, University Hospital (Inselspital) Bern, Switzerland.

Disclosure statement: The authors have declared no conflicts of interest.

Supplementary data

Supplementary data are available at Rheumatology online.

References


