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REGULAR ARTICLE

Circulating complexes between tumour necrosis factor-alpha and etanercept predict long-term efficacy of etanercept in juvenile idiopathic arthritis

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Keywords
Arthritis, Biomarker, Inflammation, Juvenile idiopathic arthritis, Tumour necrosis factor-alpha

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ABSTRACT

Aim: The relationship between tumour necrosis factor-alpha (TNF-α) and drug survival had not been studied in juvenile idiopathic arthritis (JIA), and there were no laboratory tests to predict the long-term efficacy of biological drugs for JIA. We studied whether serum levels of TNF-α, free or bound to etanercept, could predict long-term efficacy of etanercept in children with JIA.

Methods: We included 41 biologic-naïve patients with JIA who started treatment with etanercept at Skåne University Hospital between 1999 and 2010. Serum taken at the start of treatment and at the six-week follow-up were analysed for TNF-α and the long-term efficacy of etanercept was assessed using the drug survival time.

Results: Levels of TNF-α increased significantly at the six-week follow-up, and this was almost exclusively comprised of TNF-α in complex with etanercept. The increase in TNF-α showed a dose-dependent correlation to long-term drug survival ($p < 0.01$).

Conclusion: Increasing levels of circulating TNF-α at treatment initiation predicted long-term efficacy of etanercept in children with JIA, which may have been due to different pathophysiological mechanisms of inflammation. Our result may provide a helpful clinical tool, as high levels of circulating TNF-α/etanercept complexes could be used as a marker for the long-term efficacy of etanercept.

INTRODUCTION

Tumour necrosis factor-alpha (TNF-α) is believed to be a key inflammatory mediator in inflammatory joint diseases such as juvenile idiopathic arthritis (JIA) and rheumatoid arthritis (RA) (1–3). Drugs that efficiently bind and neutralise soluble TNF-α have been used to treat these conditions for nearly two decades. Since their introduction, the short-term outcome for these patients has improved dramatically, but there are fewer studies of the long-term efficacy of TNF-α inhibitors, especially in children (4–6). High levels of TNF-α in the joint are associated with increased inflammation (3), but levels of TNF-α in serum are low or undetectable in patients with RA and JIA. On the other hand, it has been shown that after the administration of TNF-α inhibitors, levels of TNF-α in the circulation initially increase. This paradox is believed to be due to the formation of TNF-α/TNF-α inhibitor complexes with prolonged survival in the circulation (7), although this has never been proved. Etanercept was one of the first available TNF-α-inhibitors. It is a fusion protein comprised of the TNF receptor 2, which binds TNF-α, anchored to the fragment crystallisable (Fc) part of an immunoglobulin G_1. This creates a molecule with prolonged survival in the circulation that efficiently neutralises soluble TNF-α, as well as TNF-β, although TNF-β is not believed to be involved in the pathogenesis of RA or JIA (8).

Abbreviations
CBA, Cytometric bead array; CRP, C-reactive protein; ELISA, Enzyme-linked immunosorbent assay; JIA, Juvenile idiopathic arthritis; RA, Rheumatoid arthritis; RF, Rheumatoid factor; TNF-α, Tumour necrosis factor-alpha.

Key notes

- There were no laboratory tests able to predict the long-term efficacy of biological drugs in juvenile idiopathic arthritis prior to this study.
- We demonstrated that high levels of tumour necrosis factor-alpha in complex with etanercept predicted the long-term efficacy of etanercept.
- This may have clinical implications, as levels of tumour necrosis factor-alpha in complex with etanercept could be used as a prognostic marker for long-term drug survival.

JIA is defined as the onset of arthritis in a child younger than 16 years of age where no other causes of arthritis can be identified. Specific drug response criteria are often used when evaluating short-term drug efficacy and long-term drug efficacy (9–12). These criteria are well documented and valid when assessing short-term responses to TNF-α inhibitors. An alternative method of evaluating drug responses is drug survival time. Drug survival time is a composite measure, integrating, among other things, both efficacy and tolerance to therapy. Drug survival has been used in several studies and has been shown to be a clinically relevant measurement for long-term drug efficacy (13,14).

The short-term response to anti-TNF-α treatment in JIA varies among individuals, but approximately one-third of the patients are good responders, one-third are intermediate responders, and one-third are nonresponders (15). Prognostic factors for good responses in children include low Childhood Health Assessment Questionnaire scores, young age and male sex (15,16).

There are only a few studies that have focused on biomarkers for drug efficacy in JIA. Serum levels of calprotectin, also known as S100A8/A9 or MRP8/14, have been shown to correlate to short-term responses to therapy with TNF-α inhibitors (17), as well as methotrexate (18). Other studies have shown that levels of circulating etanercept (19) and levels of TNF-α in synovial tissue (20) predict short-term responses in RA. Neither of these two studies addressed long-term efficacy or included children with JIA.

Our objective was to study whether levels of TNF-α were elevated after treatment with etanercept in children with JIA, as has been shown in RA, and, if so, whether the increase in TNF-α in serum comprised free TNF-α or TNF-α bound to etanercept. Furthermore, we speculated that those with an increase in TNF-α, bound to etanercept, could represent a subgroup of patients with a disease mainly driven by TNF-α. Therefore, we finally set out to investigate whether serum levels of TNF-α bound to etanercept could predict long-term efficacy of etanercept treatment in children with JIA.

METHODS

Patients

We identified 53 biologic-naïve patients with nonsystemic JIA who started treatment with etanercept at the Department of Pediatric Rheumatology, Skåne University Hospital, between 1999 and 2010. The patients were classified according to the International League of Associations for Rheumatology classification criteria for JIA into persistent and extended oligoarthritis, RF-negative polyarthritis, RF-positive polyarthritis, enthesitis-related arthritis, psoriatic arthritis and undifferentiated arthritis (Table 1) (21). Of the 53 children, 12 were excluded due to the lack of appropriate serum samples. These children did not differ in age, gender or subtype of JIA from the rest: four were oligoarticular, two were RF-negative polyarticular, one was RF-positive polyarticular, three were enthesitis-related arthritis and two were undifferentiated.

Table 1 Patient data, concomitant drugs and follow-up time

Sex	33 female, 8 male
Age at diagnosis [median (min–max)]	5.5 years (1–16)
Time to etanercept [median (min–max)]	3.6 years (0.4–13.2)
Drug survival of etanercept [median (min–max)]	50 months (3–162)
Follow-up time [median (min–max)]	90 months (53–181.5)
Drugs and clinical data at the start of etanercept	
Prednisolone*	41%
DMARD	85%
Drugs and clinical data at six-week follow-up	
Prednisolone*	37%
DMARD	76%
Diagnosis†	
Oligoarthritis (persistent and extended)	13
RF-negative polyarthritis	13
RF-positive polyarthritis	7
Enthesitis-related arthritis	5
Undifferentiated arthritis	3
Psoriatic arthritis	0

DMARD, disease-modifying antirheumatic drug; methotrexate, sulfasalazine, hydroxychloroquine, cyclosporine.
*Two patients received betamethasone instead of prednisolone.
†Diagnosis based on ILAR classification.

The drug survival time was calculated and it was defined as the number of months that the patient received etanercept. In our cohort, no patient stopped treatment due to adverse events, nor did any discontinue treatment due to a lack of availability of etanercept. Patients were followed regularly every third to sixth month for clinical evaluation, and blood samples were stored.

The Regional Ethics Review Board at Lund University approved the study (LU 2011/379), and all samples were taken with the informed consent of participants and their parents.

Blood sampling

Serum was collected just before starting treatment with etanercept and the follow-up, which took place at a median of six weeks and a range of six to 16 weeks after initiation of therapy. Serum was stored at −80°C until it was ready to be used. Plasma levels of C-reactive protein (CRP) were routinely measured at every visit.

Detection of TNF-α in serum by enzyme-linked immunosorbent assay and immuno-beads

The level of free and bound TNF-α was measured using a Human TNF-α US enzyme-linked immunosorbent assay (ELISA) kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. To ensure that etanercept did not interfere with the ELISA, etanercept (Pfizer, New York, NY, USA) was added in increasing concentrations (1:0.125 to 1:20, molar excess) to known concentrations of TNF-α (10 ng/mL; Invitrogen) and subsequently analysed

for TNF-α. No interference of etanercept on the levels of TNF-α was detected (data not shown).

Thus, we concluded that the ELISA identified free TNF-α and TNF-α bound to etanercept equally well.

To confirm the results obtained in the TNF-α-ELISA, TNF-α levels in serum were also analysed using the cytometric bead array (CBA) sensitivity kit (BD Biosciences, Erembodegem, Belgium) according to the manufacturer's instructions. FACSCanto Cytometer and FACSDiva Software (BD Biosciences) were used to detect the CBA beads. The settings were according to the manufacturer's instructions. Levels of TNF-α were calculated using FCAP Array Software version 3.0 (BD Biosciences).

Immunoadsorption of TNF-α/etanercept complexes

To analyse the ratio between free TNF-α and TNF-α in complex with etanercept, a method to immunoadsorb TNF-α/etanercept complexes with protein G was established. TNF-α at a final concentration of 10 ng/mL (Invitrogen) and etanercept at a final concentration of 500 ng/mL (Pfizer) was incubated at a molar ratio of 1:20 for 30 minutes at 37°C in order to form complexes, after which the sample was diluted 1:1 in normal, healthy serum. Patient sera were concurrently diluted 1:1 in phosphate-buffered saline (PBS). As a control, recombinant TNF-α was added separately to protein G. Samples were added to a surplus of sepharose-coupled protein G (Pharmacia, Uppsala, Sweden) to immunoadsorb complexes between TNF-α and etanercept by binding to the Fc part of etanercept. The samples were incubated with protein G for 15 minutes at room temperature followed by centrifugation for 10 minutes at 5000 g. Supernatants were removed and centrifuged for 10 minutes at 5000 g after which the new supernatants were collected. The protein G was incubated with 0.1 mol/L glycine/HCl pH 2.5 buffer, mixed and centrifuged for five minutes at 5000 g to elute the complexes. The eluate was collected and pH stabilised by Tris pH 9.0. Samples were analysed using the TNF-α ELISA as described above.

Categorising patients

To perform the subgroup analysis, the patients were classified into responders or nonresponders to anti-TNF-α therapy. Responders were defined as children in remission (n = 4), with ongoing etanercept treatment at the end of the study (n = 16) or receiving ongoing treatment when lost to follow up (n = 3). Nonresponders were defined as children who failed to respond to etanercept (n = 18).

When we performed the life-table analysis, the patients were categorised into three groups instead, based on the level of the increase in their TNF-α values. There was one group with no increase in TNF-α levels (≤0), one with a small increase in TNF-α levels (>0 to 6.0; the median value of the children with an increase) and one group with a large increase in TNF-α (>6.0; the median value of the children with an increase).

Statistics

Due to the limited number of samples, the nonparametric Wilcoxon signed-rank test was used to compare levels of TNF-α in serum taken before and after six weeks of treatment with etanercept, as well as levels of TNF-α before and after immunoadsorption and between levels after adsorption and eluate. The Mann–Whitney *U*-test was used to compare levels of TNF-α between responders and nonresponders. The log-rank test for trends was used to compare the differences between the different groups in the life-table analysis. A p-value of <0.05 was considered significant. GraphPad Prism version 6 (GraphPad Software Inc, San Diego, CA, USA) was used for the statistical analyses.

RESULTS

Patient and drug characteristics

We investigated 41 children with JIA treated with etanercept as their first biological treatment. The median age at diagnosis was 5.5 years and eight children were boys and 33 were girls. Of the 41 patients we included, 18 patients stopped treatment with etanercept due to lack of efficacy, 16 patients were still on etanercept when the study finished, four patients stopped treatment due to remission and three patients moved to other hospitals and were therefore lost to follow up. No patient stopped treatment due to adverse events. The patient data and concomitant drugs are summarised in Table 1. More detailed information is provided by Table S1.

Increased levels of bound TNF-α in serum at six-week follow-up

TNF-α levels were measured in serum taken before and after six weeks of treatment with etanercept. Levels of TNF-α were increased at the six-week follow-up (median 4.00 versus 9.52 pg/mL, $p < 0.001$) (Fig. 1). The results were confirmed using the immuno-bead assay in ten randomly selected patients (data not shown).

As it seemed unlikely that the levels of free TNF-α would increase during treatment with etanercept, we analysed whether the increase in TNF-α in serum was due to the formation of complexes between TNF-α and etanercept. Incubating serum samples, taken during the six-week follow-up, with protein G, which effectively binds etanercept via its Fc part, resulted in a marked reduction in TNF-α detectable in serum (n = 6). After eluting the protein G with glycine, the levels of TNF-α were reconstituted (Fig. S1). No binding of free TNF-α to protein G was seen. We therefore concluded that the TNF-α detected in serum at the six-week follow-up almost exclusively comprised TNF-α in complex with etanercept.

Long-term response to etanercept correlated dose dependently to increased levels of TNF-α/etanercept complexes

To investigate whether the response to therapy, measured as the drug survival time, correlated to increased levels of TNF-α/etanercept, we categorised the children into responders and nonresponders.

In the responders, levels of TNF-α were increased at the six-week follow-up (median 3.7 versus 10.4 pg/mL, $p < 0.001$). No significant increase was seen in the nonresponders (median 4.1 versus 5.5 pg/mL) (Fig. 2). No difference in

levels at the start of treatment was seen between the responders and nonresponders. However, at the six-week follow-up, responders had higher levels of TNF-α than nonresponders (median 10.4 versus 5.5 pg/mL, $p < 0.05$). Calculating the levels of the increase, by subtracting the levels of TNF-α at the six-week follow-up from the levels at the start of the treatment, showed that, as a group, the responders had a significant increase in levels of TNF-α at follow-up compared to the nonresponders (median 6.0 pg/mL, responders versus 0.7 pg/mL, nonresponders, $p < 0.01$) (Fig. 2).

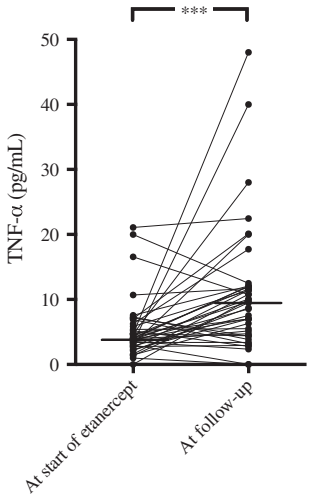

Figure 1 Levels of TNF-α before and after the start of treatment with etanercept. Levels of TNF-α were measured with ELISA in serum samples taken before the start of treatment with etanercept and at the six-week follow-up in children with JIA. The median level of TNF-α at the start was 4.00 pg/mL, whereas it was 9.52 pg/mL at follow-up ($p < 0.001$). Median values are depicted as thick lines. ***$p < 0.001$.

To further analyse the impact of TNF-α levels in predicting drug responses, life-table analyses were used. The children were categorised into three groups based on how much their TNF-α increased after initiation of treatment. The results clearly demonstrated a dose-dependent difference between the groups ($p < 0.01$, Fig. 3). Of those children who did not show an increase in TNF-α, only 36% achieved a five-year drug survival, whereas in the group with a small increase, 52% achieved a five-year drug survival. Of the children with a large increase, 84% were still receiving etanercept after five years.

As the inclusion time of this study spanned more than 11 years, during which new biological treatments were introduced and treatment recommendations may have been changed, we investigated whether the time point of etanercept initiation had an impact on the drug survival of etanercept. No such correlation was detected (data not shown).

In summary, we concluded that increased levels of TNF-α bound to etanercept after initiation of treatment with etanercept provided a prognostic marker for the long-term efficacy of the drug, measured as drug survival time.

DISCUSSION

We report that circulating levels of TNF-α were increased in children with JIA after initiation of treatment with etanercept. This circulatory increase consisted almost exclusively

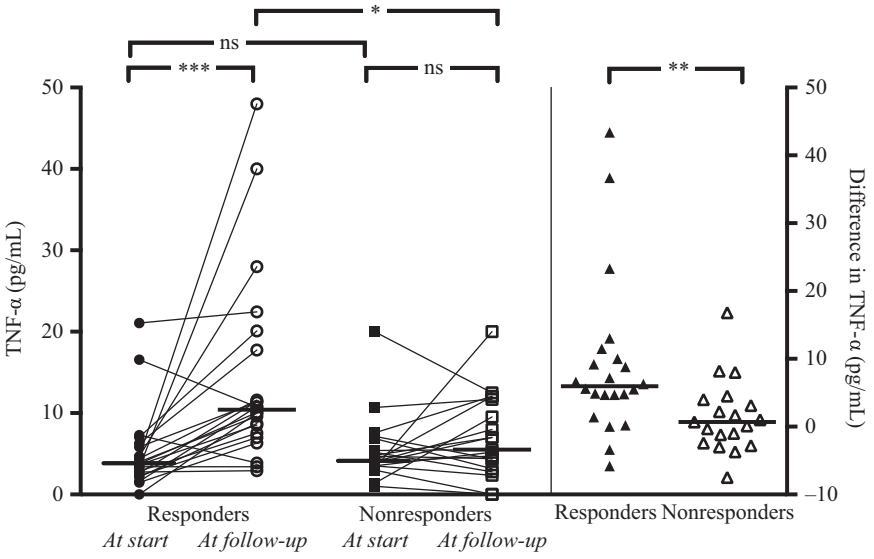

Figure 2 Levels of TNF-α in responders and nonresponders. To investigate whether the response to therapy correlated to increased levels of TNF-α/etanercept, we divided the children into responders and nonresponders. In the responders, levels of TNF-α were increased as the six-week follow-up compared to the start of treatment (median 10.4 versus 3.7 pg/mL, $p < 0.001$). No increase was seen in the nonresponders (median 5.5 versus 4.1 pg/mL). No differences in the levels at the start of treatment were seen between the responders and nonresponders. However, at the six-week follow-up, responders had higher levels of TNF-α than nonresponders (median 10.4 versus 5.5 pg/mL, $p < 0.05$). Calculating the increases in TNF-α shows that only the group of children who responded to therapy showed a significant increase in the levels of TNF-α at follow-up, compared to the nonresponders (median 6.0 pg/mL, responders versus 0.7 pg/mL, nonresponders, $p < 0.01$). Median values are depicted as thick lines. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ns, not significant.

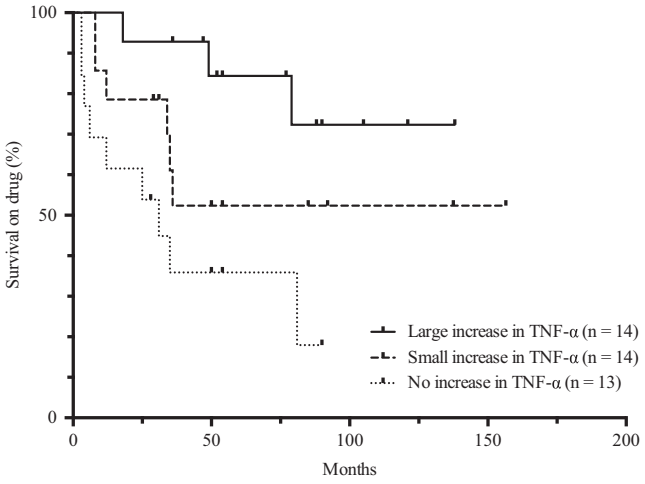

Figure 3 Drug survival in children with JIA. To further evaluate the relevance of increase in TNF-α levels and drug survival, life-table analysis was performed. The patients were divided into three subgroups, one with no increase in TNF-α, one with a small increase in TNF-α and another with a large increase. Life-table analyses of the groups were clearly different ($p < 0.01$).

of TNF-α in complex with etanercept. Studies have shown increased levels of TNF-α after initiation of anti-TNF-α therapy in both children and adults with arthritis (7,22,23). However, neither the nature of this increase or any clinically applicable correlates have, to our knowledge, been demonstrated. As only some of the patients in our study had increased levels of TNF-α/etanercept complexes in serum, we speculated that the increase in TNF-α/etanercept complexes in these children might have correlated to responses to therapy. To further study this possibility, we related the increase in TNF-α to long-term responses to therapy, measured as drug survival, a suitable long-term clinical correlate. When we divided the children into responders and nonresponders, we found that only the responders had significantly increased levels of TNF-α at the six-week follow-up. The findings were further corroborated in life-table analyses that showed a dose-dependent increase in drug survival, with higher increase in TNF-α values. Thus, we suggest that the increase in TNF-α/etanercept complexes in serum taken at the six-week follow-up in children with JIA may be used as a biomarker for the long-term drug efficacy of etanercept.

Although we demonstrate an increase in TNF-α/etanercept complexes after the onset of treatment with etanercept, the pathophysiological explanation of this phenomenon needs further investigation. One might, however, speculate that children with long-term drug survival and an increase in TNF-α comprise a subpopulation of patients with a more TNF-α-driven disease. Indeed, others have shown that in adults with RA, high levels of synovial expression of TNF-α correlate with short-term responses to infliximab, another TNF-α-inhibitor (20). One could argue that in our patients, etanercept may have bound to TNF-α locally, increased its half-life in serum and thereby accumulated TNF-α in the circulation. The amount of TNF-α measured may, thus, reflect the total amount of TNF-α produced in the joints over a period of time, although this remains speculative. It is also not clear whether the phenomenon with TNF-α/TNF-α-inhibitor complexes depends on a specific drug, etanercept, or whether it is a general phenomenon valid for all TNF-α inhibitors. Other TNF-α inhibitors might have behaved differently, which needs further investigation. In addition, JIA is composed of several subgroups with somewhat different clinical presentations, and therefore, a subgroup analysis would be of interest. However, due to the limited amount of patients in our cohort, we could not perform such an analysis.

Several different methods for measuring drug responses may be used. In this study, we chose to measure drug survival, which is an integrated measure of efficacy and tolerance. In our cohort, no patient stopped treatment due to adverse events or lack of availability of the drug, and thus, we found that drug survival in our setting was an appropriate and clinically relevant method to evaluate the long-term efficacy of etanercept. Furthermore, we could not identify any secular trends on etanercept drug survival, although our patient numbers were limited.

Studies on biomarkers for long-term responses to therapy in JIA are lacking, and there are only few studies that have focused on biomarkers for short-term drug efficacy in JIA (17,18). Many studies have, instead, focused on identifying subgroups of JIA that are more likely to respond to TNF-α inhibition. In general, children with low Childhood Health Assessment Questionnaire scores, young age and other subgroups than systemic JIA are more likely to respond to treatment (15,16). We did not specifically study these risk factors in our cohort; however, the increase in TNF-α/etanercept complexes at the six-week follow-up could predict drug survival regardless of other prognostic factors.

In this study, we demonstrate a novel method for predicting the long-term efficacy of etanercept in children with JIA by measuring the increase in TNF-α serum levels six weeks after the onset of treatment. Correlation between an increase in TNF-α and drug survival may identify a subset of patients with a more TNF-α-driven disease process. Our findings may provide a helpful clinical tool for predicting the long-term outcome and choice of drugs when treating children with JIA.

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CONFLICT OF INTEREST

The authors declare no conflict of interests.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1 TNF-α/etanercept complexes in the circulation. Serum samples (n = 6) taken at the six-week follow-up in patients with high levels of TNF-α were further analysed (A). In these serum samples etanercept, free or bound to TNF-α, was immunoadsorbed using protein G. This resulted in an almost complete loss of detectable TNF-α in the samples (B). The protein G was subsequently eluted, thus releasing all bound etanercept, resulting in reconstituted levels of detected TNF-α (C). No binding of free TNF-α to protein G was seen. We therefore concluded that the levels of TNF-α detected in the serum at follow-up were almost exclusively comprised of TNF-α in complex with etanercept and not free TNF-α. Median values are depicted as thick lines. $*p < 0.05$.

Table S1 Detailed patient data.

