Predictive value of *ex-vivo* drug-inhibited cytokine production for clinical response to biologic DMARD therapy in rheumatoid arthritis

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Abstract

Objective

To investigate ex-vivo drug-inhibited cytokine production before the start of a biological DMARD (bDMARD) as predictor of treatment response in rheumatoid arthritis (RA).

Methods

In a prospective RA cohort study [BIO-TOP], blood samples were obtained from patients before the start of a bDMARD (abatacept, adalimumab, etanercept, rituximab or tocilizumab). Peripheral blood mononuclear cells were pre-incubated for 1 hour with the therapeutic in-vivo concentration of the bDMARD and stimulated for 24 hours with heat-killed Candida albicans or Pam3Cys. Concentrations of IL-1β, IL-6, TNFα, IL-17 and IFNγ were determined by ELISA. EULAR response (good vs. moderate/no) was assessed at month 6. Area under the receiver operating characteristic curves (AUCs) were generated to evaluate the predictive value of baseline characteristics and ex-vivo cytokine production (including stimulated cytokine concentrations and absolute changes after inhibition by a bDMARD). Logistic prediction models were created to assess the added value of potential cytokine predictors.

Results

277 RA patients were included with 330 blood samples. Good response was reached in 39% of the cases. DAS28-CRP was predictive for response to adalimumab (AUC 0.70, 95%CI 0.57–0.83), etanercept (AUC 0.68, 95%CI 0.58–0.78) and rituximab (AUC 0.76, 95%CI 0.65–0.86). ACPA was modestly predictive for response to abatacept (AUC 0.63, 95%CI 0.52–0.75). In the ex-vivo analysis, 4 of 64 (6%) tests showed some predictive value but these had no added value to clinical factors routinely measured in RA, such as DAS28-CRP.

Conclusion

Ex-vivo inhibition of cytokine production by bDMARDs is unable to help prediction of treatment response to bDMARDs in RA.

Key words

rheumatoid arthritis, bDMARD, prediction of response, biomarkers, cytokines

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Introduction

Current treatment of rheumatoid arthritis (RA) consists of a "trial-and-error" approach, attempting to obtain good response with disease-modifying antirheumatic drugs (DMARDs) tried consecutively. Approximately 60% of RA patients do not achieve good clinical response after 6 months of treatment with a biological DMARD (bDMARD) (1). As a consequence, non-responding RA patients have a prolonged high disease activity which can cause worsening of physical functioning and radiographic joint damage (2, 3). It would therefore be desirable to predict, before the start of treatment, which bDMARD has the highest chance of good response in an individual RA patient. This would improve timely disease control (4).

A valuable predictor of treatment response should change the a-priori probability of 40% of good response to a bDMARD to a clearly higher or lower post-test probability. Also, the response chance should be different between bDMARDs to have clinical utility. Unfortunately, so far studies have failed to consistently identify a biomarker that can predict individual treatment response to bDMARDs with sufficient predictive value to be used in daily practice (5).

In a previous study, we demonstrated that the *ex-vivo* effects of adalimumab and golimumab on IL-1β, IL-6 and TNFα production were highly similar, while the effects of etanercept were much different (6). We suggested that this high similarity between the *ex-vivo* effects of adalimumab and golimumab might explain the much lower response to golimumab after adalimumab failure compared to etanercept failure found in RA patients in the GO-AFTER trial (7). These results also lent support to the concept that determining the *ex-vivo* effects of bDMARDs on cytokine production ("drug-inhibited cytokine production") might be a promising way to predict treatment response, since it might resemble the actual drug effect in RA patients.

The aim of this study was therefore to investigate *ex-vivo* drug-inhibited cytokine production in blood samples taken before the start of bDMARD treatment as predictor of clinical response after 6 months in RA patients.

Materials and methods

Patients

Consenting patients with RA (based on 2010 and/or 1987 American College of Rheumatology criteria and/or clinical diagnosis by the treating rheumatologist) who started or switched to a bDMARD (abatacept, adalimumab, etanercept, rituximab and tocilizumab) in the Sint Maartenskliniek (Nijmegen, the Netherlands) were included in the prospective exploratory longitudinal cohort study BIO-TOP [Biologic Individual Optimized Treatment Outcome Prediction]. These bDMARDs were chosen, since they are used most frequently and encompass the available modes of action (CTLA4-Ig fusion protein, human IgG1 anti-TNF-α monoclonal antibody, soluble dimeric TNFR2 IgG1–Fc fusion protein, anti-CD20 monoclonal antibody and anti-IL-6 receptor antibody). The study was approved by the local ethics committee (CMO region Arnhem-Nijmegen, NL47946.091.14) and a description is available in the Dutch trial register (NTR4647) (8).

Clinical assessments

Demographic-, disease- and treatment specific data were collected at baseline (just before first administration of the bDMARD) and during outpatient clinical visits performed in usual care after 3 and 6 months (±1 month). Treatment choices about starting or discontinuing DMARDs, non-steroidal anti-inflammatory drugs (NSAIDs) or glucocorticoids were left to the treating rheumatologist. Primary outcome was the European League Against Rheumatism (EULAR) response criteria (good *vs.* moderate/no response) (9).

Ex-vivo cytokine production assay

At baseline, 30mL venous blood was collected into 3 EDTA tubes. Since it has been shown that freezing peripheral blood mononuclear cells (PBMCs) affects the secretion of cytokines (10), we freshly isolated PBMCs within 24 hours by density gradient centrifugation of PBS diluted blood (1:1) over Ficoll-Paque, washed them twice with saline and suspended them in culture medium (RPMI 1640 supplemented with

2mM glutamax, 50μg/mL gentamicin, 1mM pyruvate). Cells were counted in a Coulter counter. Subsequently, 5×10^5 PBMCs in a volume of 100μL were pre-incubated in round bottom 96-well plates for 1 hour at 37°C with therapeutic in-vivo concentrations of abatacept (Css 24g/mL), adalimumab (Css 5μg/mL), etanercept (Css 5μg/mL), rituximab (Cmax 450μg/mL with 10% human pool serum) or tocilizumab (Css 20μg/mL). Human IgG was used as negative control. Thereafter, cells were stimulated with RPMI 1640+ (control), heat-killed Candida albicans (*C. albicans*) (ATCC MYA-3573 (UC820)) or Pam3Cys (TLR2 agonist) enabling robust production of cytokines. After 24 hours of stimulation supernatants were collected for the measurement of IL-1β, IL-6, TNF-α (key cytokines in the pathogenesis of RA and bDMARD targets). After 7 days of stimulation supernatants were harvested for the measurement of IL-17 and IFNγ (T cell cytokines involved in the pathogenesis of RA and affected by some bDMARDs).

Cytokine concentrations were determined by enzyme-linked immunosorbent assays (ELISA) (TNF-α, IL-1β, IL-17: R&D Systems Abingdon UK; IL-6, IFNγ: Sanquin Amsterdam the Netherlands). We chose a-priori not to measure *ex-vivo* effects of adalimumab and etanercept on TNF-α (binding of TNF inhibitors to TNF-α interferes with TNF-α detection) and IL-17 and IFNγ (both below detection limit after 24 hours). Also, we chose not to measure IL-17 and IFNγ after stimulation with Pam3Cys, since it does not induce a T cell response. Assay results were not available when disease activity was assessed, thereby preventing expectation bias. Of note, in patients who switched to a next bDMARD during the study, a blood sample was obtained again before the start of the next bDMARD.

Statistical analysis

All analyses were performed with STATA 13 statistical software. Descriptive statistics were provided with mean (standard deviation (SD)), median (interquartile ranges [p25-p75]) or frequencies depending on data distribution. Effect size calculations have shown that with 80 patients per bDMARD group and an EULAR good response of 40%, the 95% confidence interval around the point estimate of the sensitivity and specificity is ±0.13.

Absolute changes in cytokine concentrations after inhibition by each bDMARD were calculated. In total, our *ex-vivo* analysis consisted of 64 tests: 3 bDMARDs (abatacept, rituximab, tocilizumab) x (8 stimulated cytokine concentrations + 8 absolute changes in cytokine concentrations) + 2 bDMARDs (adalimumab, etanercept) x (4 stimulated cytokine concentrations + 4 absolute changes in cytokine concentrations).

Patients with a EULAR good response were termed responders and patients with an EULAR moderate or no response were termed non-responders. Discontinuation of the bDMARD before 6 months due to lack of effect was regarded as non-response and for discontinuation due to other reasons the clinical response at month 3 was carried forward.

Area under the receiver operating characteristic curves (AUCs) were generated to evaluate the predictive value of baseline characteristics (depicted in Table I) and both stimulated cytokine concentrations and absolute changes in cytokine concentrations after inhibition by a bDMARD for EULAR good response [yes *vs*. no] to the corresponding bDMARD at month 6. We did not apply a multiple testing correction, since our study had an exploratory design. For potential *ex-vivo* cytokine predictors (AUC confidence interval contains no 0.50), we performed logistic prediction modelling by adding the *ex-vivo* test to a baseline model of clinical factors and tested the equality of the 2 AUCs (11) (Fig. 1).

Results

Patient characteristics

Between June 2014 and February 2017, 277 patients were included. In total, a bDMARD was started 330 times (36 patients with 2 consecutive bDMARDs, 7 patients with 3 bDMARDs and 1 patient with 4 bDMARDs during the study). The baseline characteristics are depicted in Table I.

Table I. Baseline characteristics.

	n=330 baselines in 277 patients
Demographics	
Age, years‡	59 (12)
Female gender	226 (69%)
Disease duration, years†	7 [2-15]
RF positive	212 / 325 (65%)
ACPA positive	198 / 306 (65%)
Erosive	154 (47%)
Disease characteristics	
DAS28-CRP‡	3.9 (1.1)
TJC†	4 [2-8]
SJC†	3 [1-6]
PGA, VAS 0-100mm†	65 [50-80]
CRP, mg/L†	7 [1-25]
ESR, mm/h†	19 [10-36]
Treatment characteristics	
n. of previous bDMARDs	
0	131 (40%)
1	76 (23%)
2	66 (20%)
>2	57 (17%)
Starting bDMARD at baseline	
abatacept	25 (8%)
adalimumab	62 (19%)
etanercept	117 (35%)
rituximab	88 (27%)
tocilizumab	38 (11%)
Concomitant treatment use	
csDMARDs	225 (68%)
MTX	150 (45%)
NSAIDs	203 (62%)
Oral glucocorticoids	83 (25%)

Data presented as number (%) unless otherwise noted. ‡Mean (SD). †Median [p25-p75].
Missing data: PGA is missing in 7 cases (2%) and ESR is missing in 24 cases (7%).
If PGA was missing, DAS28-CRP was calculated with three variables: TJC, SJC and CRP.
ACPA: anti-citrullinated protein antibodies; bDMARD: biological disease-modifying antirheumatic drug; CRP; C-reactive protein; csDMARD: conventional synthetic DMARD; DAS28-CRP; 28-joint count Disease Activity Score using CRP; ESR: erythrocyte sedimentation rate; MTX: methotrexate; NSAID: nonsteroidal anti-inflammatory drug; PGA: Patient global disease assessment; RF: rheumatoid factor; SJC; swollen joint count; TJC: tender joint count; VAS: visual analogue scale.

Baseline ex-vivo cytokine concentrations

Almost all RPMI values were below detection limit, indicating good baseline control quality.

Pre-incubation with either abatacept, adalimumab, etanercept, rituximab or tocilizumab resulted generally in decreased or unchanged *C. albicans*-induced and Pam3Cys-induced cytokine productions (Supplementary file 1).

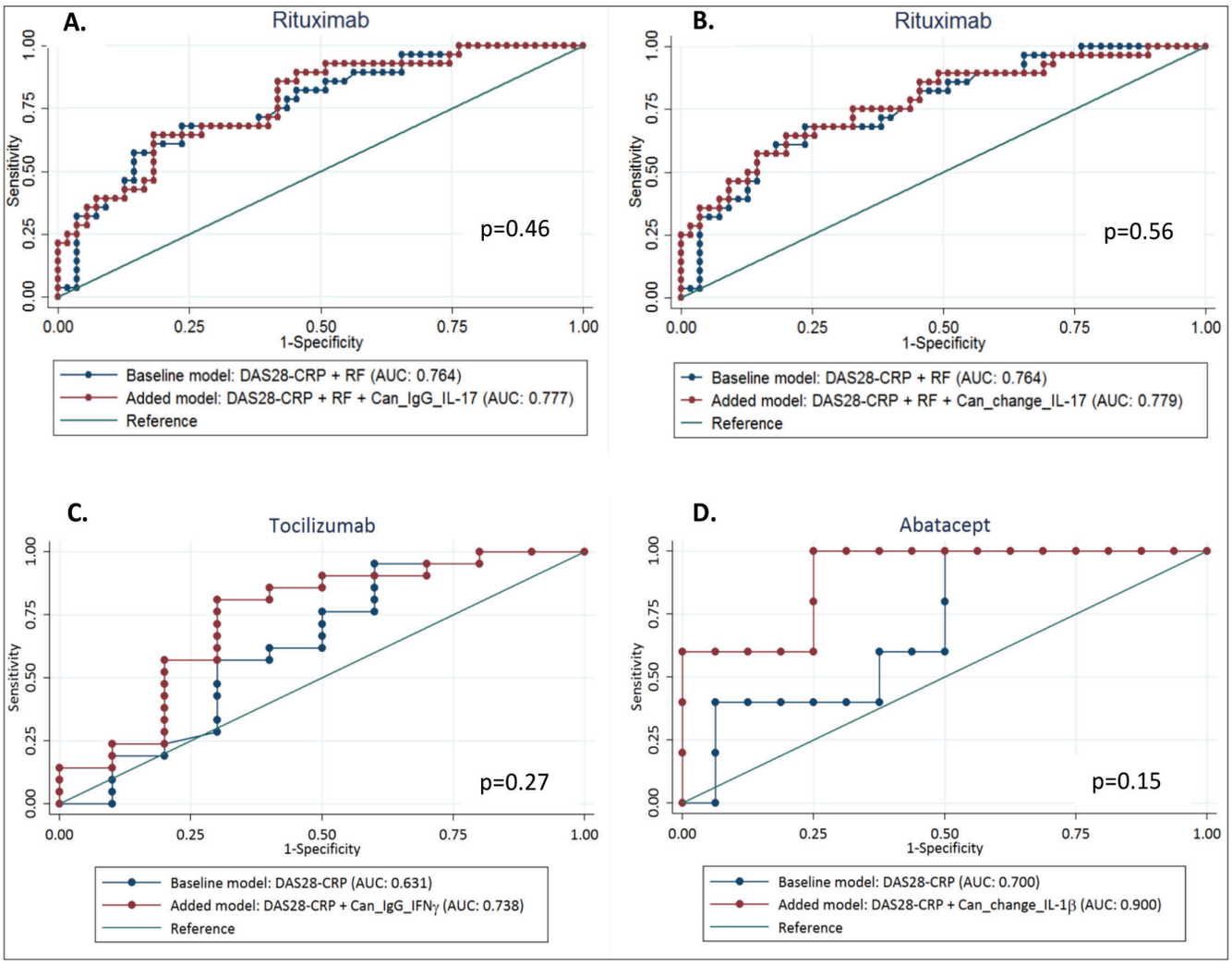

Fig. 1. Added predictive value of potential *ex-vivo* cytokine predictors for treatment response to a bDMARD.
For potential *ex-vivo* cytokine predictors (AUC confidence interval contains no 0.50), we performed logistic prediction modelling. By univariate logistic regression analyses, baseline characteristics that were associated with treatment response were identified. These were entered in a multivariate logistic regression analysis using stepwise backwards selection to construct a baseline prediction model. The potential *ex-vivo* predictor was added to the baseline model and we tested the equality of the 2 AUCs (22).
A. Can_IgG_IL-17: C. albicans-induced IL-17 production for response to rituximab (n=83).
B. Can_change_IL-17: change in C. albicans-induced IL-17 production after inhibition by rituximab for response to rituximab (n=83).
C. Can_IgG_IFNγ: C. albicans-induced IFNγ production for response to tocilizumab (n=31).
D. Can_change_IL-1β: change in C. albicans-induced IL-1β production after inhibition by abatacept for response to abatacept (n=21)
DAS28-CRP, 28-joint count Disease Activity Score using C-reactive protein; RF, rheumatoid factor [yes *vs*. no].

Clinical response to bDMARD treatment

From 23 of 330 (7%) samples the corresponding treatment response to the bDMARD could not be defined due to respectively treatment duration <2 months (n=20) and bDMARD discontinuation at month 3 due to adverse events with no DAS28-CRP measured at month 3 (n=3). In 120 of 307 (39%) cases bDMARD treatment resulted in EULAR good response at month 6. The percentage of EULAR good responders varied between bDMARDs (following order represents bDMARD preference policy at our hospital): etanercept 50/112 (45%), adalimumab 16/56 (29%), rituximab 28/86 (33%), abatacept 5/21 (24%) and tocilizumab 21/32 (66%).

Predictive value of baseline characteristics for treatment response to a bDMARD

All baseline characteristics from Table 1 were tested for their predictive value for EULAR good response to treatment with a bDMARD. DAS28-CRP was predictive for response to adalimumab (AUC 0.69, 95%CI 0.56–0.83), etanercept (AUC 0.68, 95%CI 0.58–0.78) and rituximab (AUC 0.76, 95%CI 0.65–0.86). It also tended to be predictive in abatacept (AUC 0.70, 95%CI 0.45–0.95) and tocilizumab (AUC 0.66, 95%CI 0.43–0.90), but these sample sizes were smaller. Anti-citrullinated protein antibodies (ACPA) status was modestly predictive for response to abatacept (AUC 0.63, 95%CI 0.52–0.75) and rheumatoid factor (RF) tended to be predictive for response to abatacept (AUC 0.59, 95%CI 0.49–0.69) and rituximab (AUC 0.59, 95%CI 0.48–0.69).

Predictive value of ex-vivo cytokine production for treatment response to a bDMARD

In the *ex-vivo* cytokine analysis, 4 of 64 (6%) tests did not contain 0.50 in the AUC confidence interval: *C. albicans*-induced IL-17 production for response to rituximab (AUC 0.67, 95%CI 0.54–0.80), change in *C. albicans*-induced IL-17 production after inhibition by rituximab for response to rituximab (AUC 0.35, 95%CI 0.21–0.49), change in *C. albicans*-induced IL-1β production after inhibition by abatacept for response to abatacept (AUC 0.85, 95%CI 0.60–1.00) and *C. albicans*-induced IFNγ production for response to tocilizumab (AUC 0.18, 95%CI 0.03–0.33) (Supplementary file 2). However, the prediction models of the 4 potential cytokine predictors performed similarly to their baseline models (Fig. 1).

Discussion

To our knowledge, this is the first study in which the *ex-vivo* effects of bDMARDs on stimulated cytokine production of isolated PBMCs from RA patients has been investigated.

The internal validity of our study seems to be good. The percentage of EULAR good response to a bDMARD in our study (39%) closely corresponds with the previously described percentage of 40% in literature (1). The chance of EULAR good response was lower for bDMARDs that were given in a later treatment stage (except for tocilizumab), possibly due to the selection of patients who are more refractory to treatment.

In our *ex-vivo* cytokine analysis, 4 of 64 tests showed some predictive value for treatment response to a bDMARD. This is a low number, since it is close to the 1 in 20 chance of test positivity due to chance. Two of the 4 potential *ex-vivo* predictors concerned IL-17 production for response to rituximab (*C. albicans*-induced IL-17 production and a decrease in IL-17 production by rituximab). It has been previously reported that rituximab strongly reduces (≈50%) *C. albicans*-induced IL-17 production in-vitro and that this is accompanied by an improvement in DAS28-CRP (12). Our study suggests that *ex-vivo* IL-17 (change) may be predictive for clinical response to rituximab.

We were not able to validate the findings of the 4 potential *ex-vivo* predictors in a separate cohort, since the number of included patients for abatacept, rituximab and tocilizumab was too low. However, we have shown that all 4 potential *ex-vivo* predictors have no added value to clinical factors routinely measured in RA, such as DAS28-CRP.

A strength of our study is that we made the effort to examine *ex-vivo* drug-inhibited cytokine production as a predictor of bDMARD response in a large group of RA patients treated in daily practice. Another strength is that the baseline characteristics which showed predictive value in our study were in line with previous studies [DAS28-CRP for all bDMARDs (13), ACPA for abatacept (14) and RF for rituximab (5)].

A limitation of our study is the suboptimal inclusion for abatacept, adalimumab and tocilizumab which is a consequence of the preference policy of bDMARDs at our hospital. Also, we have limited our assay to the measurement of cytokines produced by PBMCs. Although the vast majority of cytokines *in-vivo* are derived from PBMCs, some other cells can also produce them (for example IL-6 by fibroblast-like synoviocytes (15)).

Moreover, external clinical parameters might have influenced the *ex-vivo* assay such as inflammatory marker levels and concomitant treatments. However, in the designing phase of this study we decided not to use strict exclusion criteria since we wanted our cohort to reflect the general RA population.

In conclusion, we postulated that predictive biomarkers for bDMARD treatment would most likely be derived from differences in modes of action of bDMARDs since the chance of good response should differ between bDMARDs to have clinical utility. Measuring the effects of bDMARDs on cytokine production as a predictor for clinical response seemed therefore promising. However, our pragmatically designed *ex-vivo* assay is unable to help in the prediction of treatment response to bDMARDs in daily practice, since it yields only a few potential cytokine predictors and these have no added value to already used disease activity measures.

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