

Preferential decrease in IgG4 anti-citrullinated protein antibodies during treatment with tumour necrosis factor blocking agents in patients with rheumatoid arthritis

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ABSTRACT

Objective: To investigate the dynamics of IgG1 and IgG4 anti-citrullinated protein antibody (ACPA) subclasses during anti-tumour necrosis factor (TNF) treatment in patients with rheumatoid arthritis (RA).

Methods: IgG, IgG1 and IgG4 ACPA levels were determined by ELISA on anti-citrullinated fibrinogen (ACF) and IgG1 : IgG4 ACPA ratios were calculated. A pilot study was performed in 28 ACF-positive patients treated with infliximab for one year. Confirmation of the results was obtained using a cohort of 180 consecutive patients treated with adalimumab for 28 weeks.

Results: The median reduction in ACF levels was 31% for total IgG, 29% for IgG1, 40% for IgG4 and 22% for the IgG4 : IgG1 ACF ratio in the infliximab cohort. In adalimumab-treated patients, ACF levels declined 14% for total IgG and IgG1, and 36% for IgG4 ACF; the IgG4 : IgG1 ratio was reduced by 24% (all percentage values $p < 0.05$). The decrease in antibody levels was correlated with the clinical response; European League Against Rheumatism good responders had the greatest decline in antibody levels and this effect was most pronounced for IgG4 (48% reduction). The IgG4 : IgG1 ACF ratio preferentially decreased in patients with adequate therapeutic adalimumab levels.

Conclusion: ACPA subclass distribution is modulated by effective anti-inflammatory treatment. The preferential decline of IgG4 ACPA, reflected by the decreased IgG4 : IgG1 ratio, suggests a beneficial effect of anti-TNF treatment on chronic antigenic stimulation by citrullinated proteins. This effect may be directly anti-TNF mediated or the result of effective dampening of the inflammation in the rheumatoid joint.

Rheumatoid arthritis (RA) is a chronic inflammatory disease, which may lead to joint destruction.¹ One of the characteristics of the disease is the presence of autoantibodies. Anti-citrullinated protein antibodies (ACPA) comprise a group of antibodies highly specific for RA; among those described are antibodies against cyclic citrullinated peptide,² citrullinated fibrinogen,³ citrullinated alpha-enolase⁴ and mutated citrullinated vimentin.⁵ They share a similar high sensitivity and specificity for RA and are present in early and even preclinical disease.^{6,7} A pathophysiological role for ACPA in RA has been suggested,⁸ and indeed, in a serum transfer model of collagen-induced arthritis, ACPA have been shown to enhance arthritis.⁹

Two papers have recently reported on ACPA IgG subclass distribution, with similar results.^{10,11} Both show IgG1 as the main IgG subclass, as expected in a T-helper cell type 1-driven disease. Unexpectedly, IgG4 anti-citrullinated fibrinogen (ACF) and anti-cyclic citrullinated peptide antibodies were the second most frequent IgG subclass. In the latter paper, differences in isotype usage have been implied in the transition of undifferentiated arthritis to RA.¹¹ One explanation for the high frequency of IgG4 ACPA in RA might be that during prolonged antigenic stimulation a shift in the IgG4 : IgG1 antibody ratio occurs that finally results in an IgG4-dominated response.¹²⁻¹⁶

The introduction of anti-TNF agents has revolutionised RA treatment, effectively dampening inflammation in the rheumatic joint.¹⁷ Despite its proposed pathophysiological role in RA, data on the effect of anti-TNF treatment on ACPA levels in RA are controversial, because most studies reported modest or no effect of anti-TNF treatment on IgG ACPA levels,¹⁸⁻²⁶ and data on the dynamics of IgG4 and IgG1 ACPA subclasses are lacking.

If the presence of IgG4, in the context of IgG1, is a measure of chronic antigenic stimulation, as proposed by Aalberse *et al*,¹³ a reduction of chronic antigenic stimulation may lead to a preferential decrease of IgG4 ACPA. As both IgG1 and IgG4 ACPA levels might be affected by anti-TNF treatment, the relative contribution of both subclasses in an individual patient can best be studied by calculating the IgG4 : IgG1 ratio.

The aim of the present study was to investigate the dynamics of predominant ACPA subclasses during anti-TNF treatment and to relate the changes in ACPA levels to treatment response.

METHODS

Patients

Two prospective observational study cohorts were used in the present study. The first cohort consisted of consecutive RA patients treated with infliximab for at least one year at Slotervaart Hospital, Amsterdam, The Netherlands. Twenty-eight of 51 ACF-positive patients described previously had serum available for further analyses (nine non-responders, 15 moderate responders and four good responders after 46 weeks of treatment).²⁶ Serum was collected on the morning

Table 1 Baseline characteristics of the 180 patients treated with adalimumab*

Female sex, no of patients (%)	142 (79%)
Age in years, mean (SD)	53 (12)
Disease duration in years, median (IQR)	10 (4–18)
ESR in mm/h, median (IQR)	25 (11–46)
CRP in mg/l, median (IQR)	12 (6–30)
Baseline DAS, mean (SD)	5.1 (1.2)
Erosive disease, no of patients (%)	146 (81%)
Nodular disease, no of patients (%)	49 (27%)
Methotrexate use, no of patients (%)	136 (75%)
Methotrexate dose in mg, median (IQR)	20 (7–25)
ACF IgG positive, no of patients (%)	149 (83%)
ACF IgG1 positive, no of IgG-positive patients (%)	149 (100%)
ACF IgG4 positive, no of IgG-positive patients (%)	108 (79%)

ACF, anti-citrullinated fibrinogen; CRP, C-reactive protein; DAS, disease activity score; ESR, erythrocyte sedimentation rate; IQR, interquartile range.

before each infusion and stored immediately at -20°C . Infliximab was administered intravenously at a starting dose of 3 mg/kg; infusions were given at 0, 2, 6 and 14 weeks and subsequently at 8-week intervals. In patients with inadequate response, as judged by the patient's rheumatologist, the dosage of infliximab could be increased to 7.5 mg/kg ($n = 5$).

The second cohort comprised 180 consecutive patients with RA treated with adalimumab at the Department of Rheumatology of the Jan van Breemen Instituut, Amsterdam, The Netherlands. Patients were treated with either adalimumab and concomitant disease-modifying antirheumatic drugs (DMARD) or adalimumab alone (most frequently methotrexate and adalimumab; 136/180 or 75% of patients). All patients used adalimumab 40 mg subcutaneously every other week. In patients with an inadequate response, as judged by the treating rheumatologist, the dosing frequency of adalimumab could be increased to 40 mg a week ($n = 10$; seven non-responders, two moderate and one good responder at the visit before dose increases; this last observation was carried forward). Serum samples were collected just before the first injection with adalimumab at baseline, and at 4, 16 and 28 weeks. The primary analysis was performed using the samples obtained at baseline and after 28 weeks of treatment. Five patients had discontinued treatment after at least 4 weeks and 10 after at least 16 weeks of follow-up. Among these 15 patients, six were non-responders, seven moderate responders and two were good responders. In these patients, the last observation was carried forward.

All patients fulfilled the American College of Rheumatology 1987 revised criteria for RA and had active disease, indicated by a disease activity score in 28 joints (DAS28) of more than 3.2 despite earlier treatment with two DMARD including methotrexate at a dose of 25 mg a week or at the maximal tolerable dose, according to the Dutch consensus statement on the initiation and continuation of TNF blocking therapy in RA.²⁷ The study was approved by the local medical ethics committee. All patients gave written informed consent.

Clinical response

Disease activity was assessed at baseline and after 4, 16 and 28 weeks of treatment using the DAS28 score in the adalimumab cohort and during each infusion in the infliximab cohort. Clinical response was assessed by the European League Against Rheumatism (EULAR) criteria.²⁸

Antibody measurements

ACPA IgG antibodies were detected using the ACF ELISA as described previously.²⁹ For IgG1 and IgG4 ACPA subclass measurements using this IgG ACF ELISA, the optimal concentration for the mouse, anti-human (MH) monoclonal antibodies MH161-1 (anti-IgG1, clone HP 6188; Sanquin, Amsterdam, The Netherlands) and MH164-4 (anti-IgG4, clone HP 6196, Sanquin) were determined using IgG subclass-specific M proteins. The monoclonal antibodies used have been evaluated for their specificity in an International Union of Immunological Societies/World Health Organization collaborative study.^{30–31} The specificity of the anti-IgG1 and anti-IgG4 subclass monoclonal antibodies was confirmed using IgG4 and IgG1 subclass M protein as a coat and different dilutions of the horseradish peroxidase-labelled monoclonal antibody as the detecting antibody. Virtually no reactivity was seen with the anti-IgG4 monoclonal antibody on the IgG1 coat and the anti-IgG1 monoclonal antibody on the IgG4 coat (data not shown). Using a 1 : 1 mixture of IgG1 and IgG4 M protein as a coat, optimal dilutions for the monoclonal antibodies were obtained. One $\mu\text{g}/\text{ml}$ anti-IgG1 and anti-IgG4 gave comparable results to a concentration of 0.4 $\mu\text{g}/\text{ml}$ anti-IgG used in the ACF ELISA (see supplementary fig 1 available online only).

Sera were titrated in four threefold dilutions, starting at 1 : 50. The antibody concentrations were expressed in arbitrary units per millilitre (AU/ml) using the previously described reference serum²⁹ and were calibrated on the linear part of the calibrator curve. The linear part of the calibrator curves of the three conjugates was parallel (see supplementary fig 2 available online only). Total IgG ACF was defined as 1000 AU/ml. As the concentration of IgG1 is much higher than IgG4, the standard was arbitrarily defined as containing 1000 AU/ml for IgG1 and 100 AU/ml for IgG4. Coefficients of intra and interassay variation were below 20% both for the same batch of citrullinated fibrinogen and for different batches. Cut-off values for the presence of IgG1 and IgG4 subclasses of ACF antibodies were defined as the mean plus 2 SD for serum samples obtained from a group of 40 IgG ACF-negative healthy laboratory workers. This definition resulted in cut-off values for positivity of 73 AU/ml for IgG1 and 17 AU/ml for IgG4, respectively (see supplementary fig 3 available online only). In the adalimumab cohort, therapeutic adalimumab levels were measured by ELISA and anti-adalimumab antibodies were measured using a radioimmunoassay, both as described previously.³²

Statistical analysis

The analyses were performed on both cohorts separately using SPSS version 15.0. Antibody levels were analysed in the positive patients for each test only. Friedman's non-parametric repeated measures comparisons (infliximab cohort) or paired sample t test (adalimumab cohort) was used to detect changes in ACF subclass levels in time. A general linear model univariate analysis with post-hoc Bonferroni test for multiple comparisons was used to compare the relative change in antibody levels among the three EULAR response groups. As the arbitrary units were obtained from parallel calibrator curves, the IgG4 : IgG1 ratio could be calculated in patients positive for both subclasses. Log transformation to gain normality was applied when necessary. Geometric means and 95% CI were reported unless otherwise stated. Pearson's correlation coefficient was used to correlate two normally distributed variables.

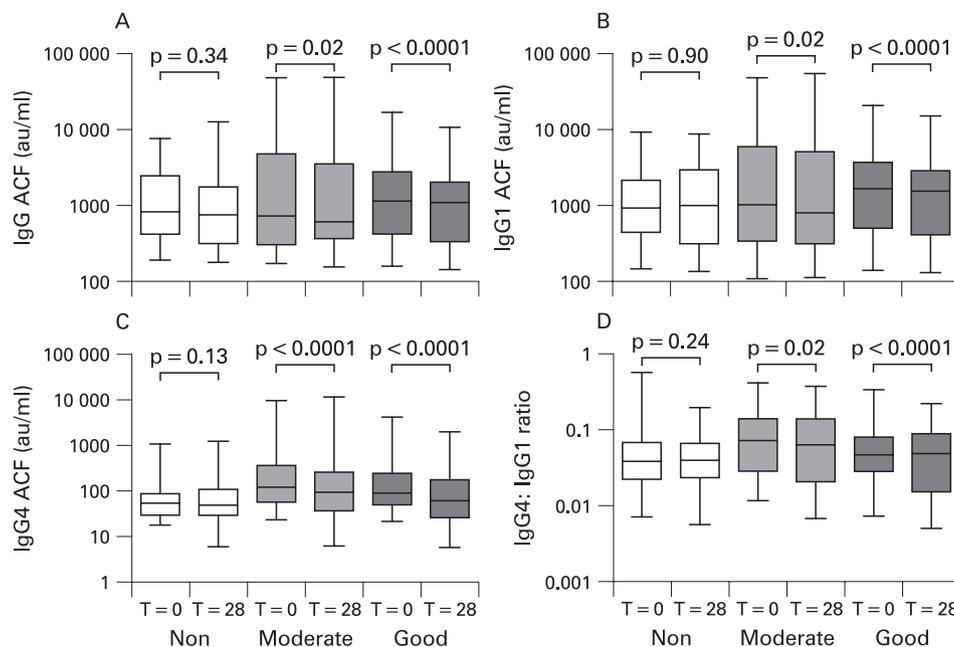


Figure 1 Antibodies to citrullinated fibrinogen response to adalimumab treatment, stratified for European League Against Rheumatism response. (A) IgG anti-citrullinated fibrinogen (ACF) mean* values at baseline and at 28 weeks were 988 arbitrary units (AU/ml (95% CI 118 to 8297) and 905 AU/ml (69 to 11 812) for non-responders, 1203 AU/ml (52 to 27 980) and 1078 AU/ml (38 to 30 646) for moderate responders and 1284 AU/ml (91 to 18 049) and 1017 AU/ml (69 to 11 812) for good responders, respectively. (B) IgG1 ACF mean* values at baseline and at 28 weeks were 1027 AU/ml (108 to 9800) and 1011 AU/ml (78 to 13 195) for non-responders, 1340 AU/ml (48 to 37 323) and 1194 AU/ml (42 to 33 922) for moderate responders and 1653 AU/ml (99 to 27 708) and 1264 AU/ml (85 to 18 692) for good responders, respectively. (C) IgG4 ACF mean* values at baseline and at 28 weeks were 64 AU/ml (10 to 427) and 53 AU/ml (5 to 577) for non-responders, 174 AU/ml (6 to 5032) and 119 AU/ml (3 to 4842) for moderate responders and 138 AU/ml (8 to 2404) and 72 AU/ml (3 to 1914) for good responders, respectively. (D) IgG4 : IgG1 ACF ratio mean* values at baseline and at 28 weeks were 0.045 (0.005 to 0.396) and 0.037 (0.006 to 0.207) for non-responders, 0.066 (0.09 to 0.509) and 0.053 (0.005 to 0.555) for moderate responders and 0.049 (0.006 to 0.390) and 0.035 (0.003 to 0.369) for good responders, respectively. *As these levels were not normally distributed, geometric means with the 95% CI is reported. Boxes depict the geometric mean with interquartile range, whiskers show 5th to 95th percentiles.

RESULTS

Infliximab cohort

Twenty-eight IgG ACF-positive patients were eligible for subclass analysis. All patients were positive for IgG1 ACF and 64% (n = 18) of the patients were positive for IgG4 ACF. Total IgG, IgG1 and IgG4 ACF antibody levels decreased significantly during the study period. The median IgG ACF levels decreased from 1182 AU/ml (interquartile range (IQR) 679–3058) at baseline, 1203 AU/ml (IQR 580–2468) at 14 weeks to 849 AU/ml (IQR 426–2410) at 46 weeks (p = 0.001). IgG1 ACF levels decreased from 1320 AU/ml (IQR 679–1321) at baseline, 1374 AU/ml (IQR 577–2686) at 14 weeks to 994 AU/ml (IQR 483–1991) at 46 weeks (p = 0.007). IgG4 ACF levels also decreased, the median levels were 162 AU/ml (IQR 122–442) at baseline and 108 AU/ml (IQR 72–223) and 96 AU/ml (IQR 48–227) at 14 and 46 weeks, respectively (p = 0.005).

In terms of percentages, the median decrease from baseline values at 46 weeks was 31% for IgG, 29% for IgG1 and 40% for IgG4. The IgG4 : IgG1 ratio decreased with 25% at 14 weeks and 22% at 46 weeks (p = 0.03). Only two patients became negative for total IgG ACF, one of those also became negative for IgG1. One other patient became negative for IgG4 ACF levels.

Adalimumab cohort

Baseline characteristics

To substantiate the findings and to investigate whether treatment response was correlated with changes in antibody levels, total IgG, IgG1 and IgG4 ACF levels were measured in 180 patients treated with adalimumab, 39 non-responders, 76 moderate and 65 good responders after 28 weeks of treatment. Table 1 shows the baseline characteristics for the adalimumab-treated patients.

Table 2 Percentage of baseline antibodies to deiminated fibrinogen (ACF) levels after 28 weeks of adalimumab treatment, stratified for EULAR response*

ACF†	All	p Value‡	Non-responders	Moderate responders	Good responders	p Value§
ACF IgG	86% (40 to 187)	0.000	92% (35 to 237)	90% (43 to 188)	79% (39 to 161)	0.140
ACF IgG1	86% (34 to 214)	0.000	99% (28 to 352)	89% (42 to 189)	77% (33 to 179)	0.040
ACF IgG4	64% (17 to 250)	0.000	83% (28 to 245)	69% (19 to 245)	52% (12 to 231)	0.022¶
Ratio IgG4 : IgG1 ACF	76% (22 to 271)	0.000	80% (15 to 244)	79% (25 to 254)	70% (23 to 223)	0.670

*European League Against Rheumatism (EULAR) response as defined by the EULAR response criteria. †Log transformed to gain normality; geometric mean and 95% CI are reported. ‡p Values compared with baseline: one sample t test. §p Values comparing responder groups: univariate analysis of variance with post-hoc Bonferroni (¶p<0.05 for non versus good responders). ACF, anti-citrullinated fibrinogen.

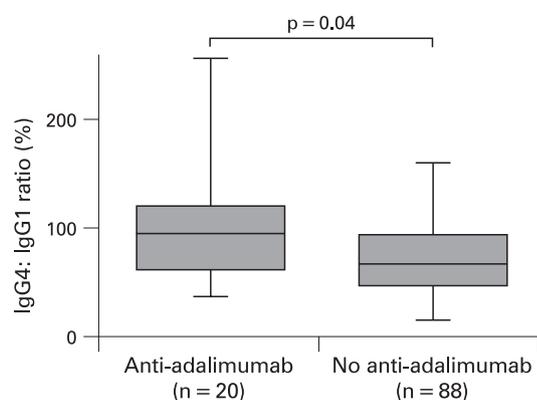


Figure 2 Percentage of baseline IgG4 : IgG1 anti-citrullinated fibrinogen ratio* after 28 weeks of adalimumab treatment, stratified for the presence of anti-adalimumab antibodies. Mean* IgG4 : IgG1 ratio at baseline and at 28 weeks was 72% (95% CI 21 to 248) and 100% (95% CI 27 to 367) for patients without and with anti-adalimumab antibodies. *As this ratio was not normally distributed, geometric mean with 95% CI are reported. Boxes depict geometric mean with interquartile range, whiskers show 5th to 95th percentiles.

Eighty-three per cent of patients were positive for total IgG ACF antibodies, all of those were also positive for IgG1 ACF and 73% of these patients were positive for IgG4 ACF. Baseline IgG4 ACF levels were associated with treatment response, although post-hoc Bonferroni analysis only showed significance between moderate and non-responders ($p = 0.03$). Baseline IgG and IgG1 levels, as well as the IgG4 : IgG1 ACPA ratio, were similar among the treatment response groups (see legend to fig 1 for levels).

ACPA IgG1 and IgG4 levels decrease during anti-TNF treatment

Total IgG, IgG1 and IgG4 ACF antibody levels decreased significantly during the study period of 28 weeks. The mean IgG ACF levels decreased from 1186 AU/ml (95% CI 74 to 19 034) at baseline to 1020 AU/ml (95% CI 65 to 15 908) at 28 weeks ($p < 0.001$). IgG1 ACF levels decreased from 1377 AU/ml (95% CI 72 to 26 486) at baseline to 1181 AU/ml (95% CI 61 to 22 699) at 28 weeks ($p < 0.001$). IgG4 ACF levels also decreased, the mean levels were 129 AU/ml (95% CI 17 to 2540) at baseline and 83 AU/ml (95% CI 3 to 2213) at 28 weeks ($p < 0.001$). Only four patients became negative for total IgG ACF, one of those also became negative for IgG1. Thirteen patients became negative for IgG4 ACF levels (12%). The mean IgG4 : IgG1 ACF ratio decreased from 0.055 (95% CI 0.007 to 0.438) to 0.042 (95% CI 0.004 to 0.396; $p < 0.001$), resulting in a 24% reduction after 28 weeks of adalimumab treatment. There was no difference in change in autoantibody levels between patients treated with different doses of infliximab ($n = 5$) or adalimumab ($n = 10$; data not shown).

Decrease in antibody levels is associated with response to anti-TNF treatment

As shown in fig 1, ACF levels decreased significantly after 28 weeks in the moderate ($p = 0.02$ for total IgG and IgG1 ACF; $p < 0.001$ for IgG4 ACF) and good responder groups ($p < 0.001$ for all three antibody levels). In contrast, non-responders did not show a significant reduction in ACF levels ($p = 0.34$, 0.90 and 0.13 for total IgG, IgG1 and IgG4 ACF, respectively). The IgG4 : IgG1 ACF ratio was also significantly reduced in moderate ($p = 0.02$) and good ($p < 0.001$) responders, but not in non-responders ($p = 0.24$).

To investigate whether the changes in antibody levels were greater in moderate and good responders compared with non-responders, ratios of antibody levels at baseline and at 28 weeks were determined and expressed as percentages. The baseline antibody level was not correlated with the calculated change from baseline value (data not shown). As shown in table 2, the change in IgG1 and IgG4 ACF, but not the decrease in the IgG4 : IgG1 ratio was significantly different among the response groups.

Preferential decrease in IgG4 ACF only in the absence of anti-adalimumab antibodies

Anti-adalimumab antibodies are associated with low levels of adalimumab.³² Therefore, we expected the IgG4 : IgG1 ratio only to decrease in those patients without anti-adalimumab antibodies (when adequate levels of adalimumab are present). Indeed, as shown in fig 2, the mean IgG4 : IgG1 ratio did not change in the group of patients with anti-adalimumab antibodies (median adalimumab level 1.4 mg/l, IQR 0.0–5.1), whereas in the group of patients without anti-adalimumab antibodies (median adalimumab level 10.9 mg/l, IQR 7.6–14.5), a mean 28% decrease in the IgG4 : IgG1 ratio was observed ($p = 0.04$).

Decrease in ACPA levels is not correlated with total immunoglobulin levels

To explore the possibility that a decrease in ACPA levels is merely a reflection of a decrease in total immunoglobulin levels, IgG, IgG1 and IgG4 levels were measured in 10 patients who showed the greatest decline in ACPA levels. There was no correlation between a decline in ACPA levels and total immunoglobulin levels after 28 weeks of adalimumab treatment ($R = -0.19$, $p = 0.604$ for IgG, $R = 0.21$, $p = 0.57$ for IgG1 and $R = 0.34$, $p = 0.33$ for IgG4; see supplementary fig 4 available online only).

DISCUSSION

The aim of our study was to investigate the dynamics of IgG1 and IgG4 ACPA subclasses in RA patients treated with the TNF-blocking agents adalimumab and infliximab. The decrease in ACPA levels of all subclasses was correlated with treatment response. A preferential decline of IgG4 ACPA as reflected by a decrease in the IgG4 : IgG1 ACPA ratio was observed and was most pronounced in patients with adequate anti-TNF levels.

The frequencies of IgG4 ACPA-positive patients in our cohorts were higher than those reported by Chapuy-Regaud *et al.*¹⁰ but lower than those reported by Verpoort *et al.*¹¹ Dissimilarity in patient cohorts ((early) RA compared with established RA patients eligible for anti-TNF treatment), the different assays and corresponding determination of cut-off values may account for the variation in reported frequencies.

Our findings substantiate and extend previous data on ACPA levels in RA patients after anti-TNF treatment.^{18–26} One study reported a decrease in the presence and levels of IgG4 ACPA after 7 years of follow-up in early arthritis patients,¹¹ but detailed data on treatment strategies in these patients were lacking. The direct effect of treatment on IgG4 ACPA levels had not been studied previously.

The pronounced effect seen on IgG4 antibody levels may be a direct effect of TNF α inhibition on IgG4 production. TNF α has been shown to modulate class switch recombination because blocking TNF α antibodies interfere with the co-stimulatory signal provided by T cells needed for IL-4-induced IgG4

synthesis.³³ A reduction of specific ACPA IgG4 would then just be a reflection of a decrease in total IgG4 levels. As changes in total IgG4 and specific IgG4 were not correlated, this hypothesis seems less likely.

The differentiated response of IgG1 and IgG4 ACPA levels may also be caused by the anti-inflammatory effect of anti-TNF treatment, as is suggested by the preferential decrease in those responding to anti-TNF treatment. As long-term chronic stimulation is needed for a pronounced IgG4 response,¹⁵ IgG4 ACPA levels might reflect chronic antigenic stimulation by citrullinated proteins.⁸ A decrease in IgG4 ACPA might thus reflect the disruption of the chronic stimulation by citrullinated proteins and subsequent interaction with ACPA by effective anti-inflammatory treatment, because citrullination is inflammation dependent.^{34,35} To gain further insight into this hypothesis, it would be interesting to correlate changes in the IgG4 : IgG1 ACPA ratio with a reduction in synovial antigenic load (ie, the amount of citrullinated proteins) during anti-TNF treatment.

The differential response for IgG1 and IgG4 may provide insight into the nature of the immune response leading to the production of these antibodies. IgG1 ACPA may be predominantly produced by long-lived plasma cells, whereas IgG4 ACPA may arise by the continuous generation of short-lived plasma cells from memory B cells, a process that would be driven by persisting antigen, in this case citrullinated proteins.³⁶ Therefore, once the antigenic load is reduced through effective inflammatory treatment, IgG4 ACPA levels drop whereas IgG1 levels remain relatively stable. To substantiate this hypothesis, it would be of interest to study ACPA subclass responses in patients treated with B-cell depletion therapy. Plasma cells do not express the target antigen (CD-20) on their surface and thus rituximab may deplete short-lived plasma cells by targeting the B-cell compartment from which they arise, whereas long-lived plasma cells may be less affected. Interestingly, Thurlings *et al*³⁷ have recently shown a direct correlation between clinical response, a decrease in serum IgG ACPA levels and a lower number of synovial plasma cells after rituximab treatment, suggesting that short-lived synovial plasma cells play a role in local ACPA production.

In summary, RA patients treated with TNF-blocking agents show a reduction in ACPA subclass levels. This reduction is seen predominantly in those responding to treatment and is more pronounced for IgG4 than for IgG1. This may reflect a disruption of the chronic antigenic stimulation of ACPA in the rheumatic joint and/or a direct effect of anti-TNF treatment on IgG4 ACPA synthesis. Further research is necessary to substantiate this hypothesis.

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Patient consent: Obtained.

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