

IgG4 Production Against Adalimumab During Long Term Treatment of RA Patients

Pauline A. van Schouwenburg · Charlotte L. Krieckaert ·
Michael Nurmohamed · Margreet Hart · Theo Rispens ·
Lucien Aarden · Diana Wouters · Gerrit Jan Wolbink

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Abstract

Purpose A substantial part of rheumatoid arthritis (RA) patients is chronically treated with adalimumab. Some of these patients produce antibodies against adalimumab, which correlate with lower serum drug levels and reduced clinical response. Long term exposure to antigens may result in antigen specific IgG4 production as was demonstrated in studies on prolonged exposure to antigens such as different allergens, Factor VIII and IFN- β . Here, we investigate whether long term treatment of RA patients with the therapeutic monoclonal antibody adalimumab leads to the production of specific IgG4 antibodies.

Methods We developed radio immunoassays to detect total IgG or IgG4 against adalimumab and applied these in a cohort of 271 consecutive RA patients during 3 years of adalimumab treatment.

Results In 32 % of the 271 patients antibodies against adalimumab were detectable. IgG4 antibodies were detected in 29 % of the patients. The proportion IgG4 of total IgG against adalimumab varies widely between patients, and IgG4 was found to contribute significantly to the anti drug antibody (ADA) response in some patients.

Conclusion In the immune response against adalimumab in adalimumab-treated RA patients a considerable part of the ADA is IgG4. Although IgG4 is often considered to be harmless due to its lack of effector function, neutralization of adalimumab by IgG4 antibodies will lead to a reduced clinical response.

Keywords Adalimumab · immunogenicity · anti-TNF · IgG4

Introduction

In human serum of Caucasian adults about 4 % of total IgG is of the IgG4 isotype [1]. IgG4 is considered an odd antibody in the sense that it is the only IgG antibody that is unable to activate complement and it has a low affinity to Fc γ receptors [2, 3]. Furthermore, IgG4 is able to exchange half molecules *in vivo*, leading to bispecific antibodies that do not cross-link, and therefore result in the formation of small immune complexes [4–6]. Because of the limited potential to trigger immunological effector functions and its tendency to form small immune complexes, IgG4 is thought to have lesser effect on the clearance of antigens and play a limited role in inflammation.

Even though in general the percentage of IgG4 is low in serum, for some immune responses it has been described that antigen-specific IgG4 is the main antibody isotype produced. Already in the 1970's it was shown that chronic antigen exposure to grass pollen and bee venom results in the predominant formation of IgG4 [1, 7]. Increased allergen specific IgG4 is associated with a beneficial response to specific allergen immunotherapy in allergic patients [8, 9].

P. A. van Schouwenburg · M. Hart · T. Rispens · L. Aarden ·
D. Wouters · G. J. Wolbink
Department of Immunopathology, Sanquin Research and
Landsteiner Laboratory Academic Medical Centre,
Amsterdam, The Netherlands

C. L. Krieckaert · M. Nurmohamed · G. J. Wolbink
Jan van Breemen Research Institute | Reade,
Amsterdam, The Netherlands

P. A. van Schouwenburg (✉)
Department of Immunopathology, Sanquin Research,
Plesmanlaan 125,
1066CX, Amsterdam, The Netherlands
e-mail: p.schouwenburgvan@sanquin.nl

Chronic treatment of patients with biological agents, such as Factor VIII, interferon (IFN)- β or therapeutic monoclonal antibodies can be considered as long-term exposure to protein antigens. IgG antibody formation against biologicals is frequently reported and for some biologicals IgG4 production has been described. For example, in hemophilia A patients treated with Factor VIII it has been shown that antibody formation leads to clearance of Factor VIII and non-responsiveness to treatment. In this immune response IgG1 and IgG4 were most prominently produced [10, 11]. Also prolonged exposure to IFN- β , in multiple sclerosis patients can lead to development of IgG4 antibodies [12]. For the immune response against therapeutic monoclonal antibodies, it has been shown that antibodies of the IgG4 isotype are formed, but longterm measurements on this subject are lacking [13–15].

Adalimumab is a fully human IgG1 therapeutic monoclonal antibody directed against TNF α which is used for treatment of different inflammatory diseases such as Rheumatoid Arthritis (RA), Ankylosing Spondylitis and Crohns disease [16, 17]. Patients are chronically treated with high doses (40 mg every other week) of adalimumab. Even though adalimumab is fully human, IgG antibody production against adalimumab has been described. Importantly, antibody production was found to be linked to lower serum drug levels and reduced clinical responsiveness [18–22].

Here, we study the relative contribution of IgG4 to total anti-drug antibody (ADA) production against adalimumab and development of anti-adalimumab IgG4 antibodies over time. To this end, we tested longitudinal samples of a cohort of 271 adalimumab treated RA patients on the presence of IgG4 ADA, total ADA and adalimumab levels during 3 years of treatment.

Patients and Methods

Patients

The prospective observational cohort study consisted of 272 consecutive RA patients treated with adalimumab at the Department of Rheumatology of the Jan van Breemen Research Institute | Reade, Amsterdam as described previously by Bartelds et al. [18]. Data and sera were available of 271 patients. The number of serum samples per patient varies between two and eleven. All patients fulfilled the American College of Rheumatology 1987 revised criteria for RA, and fulfilled the criteria of the Dutch consensus statement on the initiation and continuation of TNF α blocking therapy in RA [23]. Patients were treated either with adalimumab and concomitant DMARD therapy or with adalimumab monotherapy. 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 per week. The study was approved by the Medical Ethics Committee of the Slotervaart Hospital and Reade. All patients gave written informed consent.

Measurement of Adalimumab Concentration

Functional adalimumab levels were measured by enzyme linked immunosorbent assay (ELISA) as described previously [24]. Briefly, TNF α was indirectly coated via a monoclonal antibody against TNF and adalimumab binding was detected using biotinylated adalimumab specific rabbit anti-idiotypic antibodies. Results were related to a titration curve of adalimumab in each plate. The lowest level of detection was 0.002 mg/l.

Measuring ADA by Antigen Binding Test (ABT)

The test was essentially carried out as described before [25, 26]. For short, antibodies were captured using protein A sepharose and ADA were detected using ^{125} I labeled adalimumab F(ab) $_2$ diluted in Freeze buffer (Sanquin) [27]. Antibody levels were compared to a reference serum and expressed in arbitrary units (AU). Patients were considered positive for ADA if at one or more time points ADA were above the cut-off of 12 AU [24]. The cut-off was determined as 6 SD above the average signal measured for a panel of 100 rheumatoid arthritis patients that were not yet treated with an anti-TNF therapeutic antibody. In contrast to previous studies on the immunogenicity of adalimumab from our institute [18, 24], patients were considered positive for anti-adalimumab regardless of the serum levels of adalimumab. We chose this definition in order to be able to compare the results for the different assays, but emphasize that this definition is not based on clinical practice.

Production of Anti-IgG4 Coupled Sepharose

CNBr-activated SepharoseTM 4B (GE Healthcare, Chalfont St. Giles, UK) was incubated in 1 mM HCl (pH 3.0) for 15 min. After washing three times with H $_2$ O containing 0.1 M NaHCO $_3$ and 0.5 M NaCl (pH 8.5) (coupling buffer) monoclonal anti-IgG4 (Sanquin) (100 μ g MH164-1/100 mg sepharose) was added and incubated for 4 h at 4°C in coupling buffer. Afterwards the samples were washed three times with 0.5 M glycine (pH 8.5) and then incubated for 2 h with an excess of 0.5 M glycine (pH 9.0) in coupling buffer. Afterwards the sepharose was washed three times in acidic (acetate buffer pH 4.0) and basic (borate buffer pH8.0) conditions and then rebuffered into PBS containing 0.01 M EDTA, 0.05 % NaN $_3$, 0.3 % BSA.

Measuring IgG4 Antibodies Against Adalimumab

The method used for measuring IgG4 antibodies was similar to the measurement of total ADA with one alteration. Instead of using 1 mg Sepharose-immobilized protein A, 1 mg of anti-IgG4 coupled sepharose was used. Patients were said to be positive for IgG4 anti-adalimumab if IgG4 measurement was above the cut-off of 19 AU determined as described before [28].

Purification of Total IgG4

Total IgG4 was purified from patients serum using anti-IgG4 coupled sepharose as described before [28].

Results and Discussion

Measurement of IgG and IgG4 Antibodies to Adalimumab

In this study, two assays were used to study IgG4 production in the immune response against adalimumab: the protein A antigen binding test (ABT) and the IgG4 ABT. Using purified IgG4, we have shown that both assays are able to detect IgG4 ADA equally well and that units from both assays are comparable (Fig. 1a) [28]. However, the cut off of the IgG4

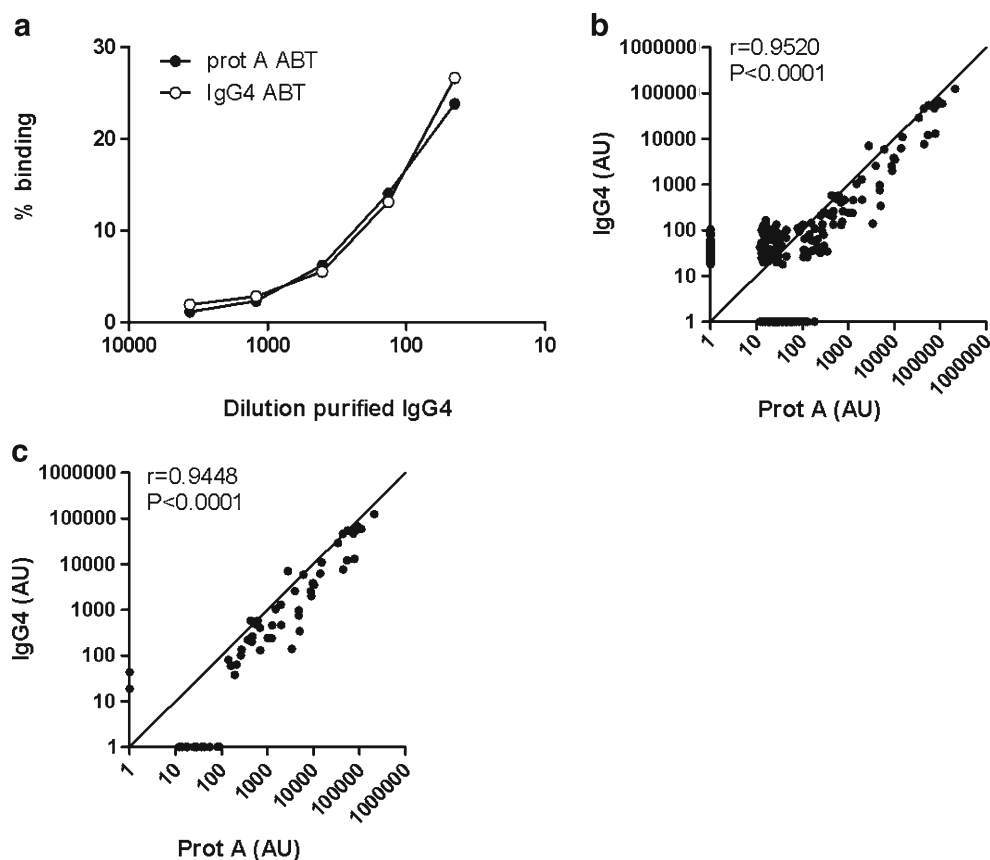
ABT is higher compared to the prot A ABT (19 AU vs 12 AU).

Relation Between Total ADA and IgG4 ADA

Total IgG and IgG4 ADA were measured in all available patient serum samples during the 3 years of follow-up using the prot A ABT and IgG4 ABT. In 62 patients both total IgG and IgG4 ADA were found. Twenty-five patients were positive for total IgG, but not for IgG4 and 17 patients were positive for IgG4 and negative for total IgG. This result can be explained by previous experiments in which we have shown that the IgG4 ABT is less sensitive for drug interference, especially in the presence of high levels of adalimumab (above 0.5 µg/ml) [28].

Figure 1b shows the correlation of all positive serum samples in either one or both assays. IgG4 anti-adalimumab levels were well correlated with total IgG production against adalimumab (pearson's test $r=0.9520$, $p<0.0001$). The data suggest that a substantial part of the ADA produced is of the IgG4 isotype. Eighty-nine patient samples from 65 patients only tested positive in the prot A ABT, and ADA levels (median and 25–75 percentile: 27 AU (14–48)) were significantly lower compared to samples that were positive in both assays (median and 25–75 percentile: 84 AU (24–574)) ($p<0.0001$, Mann–Whitney test). IgG4 may either be absent in these

Fig. 1 Correlation between total ADA and IgG4 ADA against adalimumab. **a** Purified IgG4 from patients serum, measured in both the prot A ABT and the IgG4 ABT. **b** The correlation of anti-adalimumab antibodies measured in the prot A ABT and IgG4 ABT. All positive data of a cohort of 271 patients during 3 years of treatment are plotted. All negative measurements were plotted as 1 AU. The line shows the levels at which all antibodies are of the IgG4 subtype. IgG4 significantly correlated with total IgG ($r=0.9520$, $P<0.0001$). **c** The correlation of total ADA and IgG4 antibodies against adalimumab in all sera with undetectable adalimumab levels ($r=0.9448$, $P<0.0001$)



samples or simply below the detection limit of the assay. Of these 65 patients single positive in the prot A ABT, 47 patients developed IgG4 ADA at other time points during adalimumab treatment.

Arbitrary units as measured in the prot A ABT and IgG4 ABT are comparable and IgG4 to adalimumab is detected equally well in both assays. However, an odd discrepancy in the lower area of the correlation graph can be observed. First, 78 patient samples were only positive in the IgG4 ABT. Also in these samples, IgG4 ADA levels (median and 25–75 percentile: 32 AU (27–44)) were significantly lower than in double-positive samples (median and 25–75 percentile: 72 AU (36–300)) ($p < 0.005$, Mann–Whitney test). Second, in samples with low or intermediate ADA levels, IgG4 levels appear to be higher than total IgG levels, which is by definition impossible. Both observations can be explained by the difference in susceptibility for drug interference between the prot A ABT and IgG4 ABT.

To estimate what percentage of total anti-adalimumab IgG consists of IgG4, we focused on the samples with no detectable adalimumab levels, since in these samples the influence of the difference in drug interference is expected to be limited. Figure 1c shows the correlation between total ADA levels and IgG4 ADA levels in all patient sera with undetectable adalimumab. Indeed the majority of IgG4 positive, total IgG negative can be explained by the presence of adalimumab in the patient's serum. In the two remaining points the detection of IgG4 might be the result of high levels of immune complexes in these patients which can be partly detected in the IgG4 ABT, but not the prot A ABT. Furthermore, Fig. 1c shows that the contribution of IgG4 to the total amount of adalimumab specific IgG in these sera is substantial but varies widely (median: 38 %; 25–75 percentile: 21–67 %). Unpublished data suggest that the remaining IgG produced is mainly of the IgG1 isotype and that IgG2 and IgG3 are only produced in very low numbers.

It would be very interesting to link the percentage of IgG4 in patients to adalimumab levels in the serum or clinical outcome in these patients. However, because of the difference if drug interference between the two assays the percentage in IgG4 is overestimated in samples with high adalimumab titers. Therefore these type of analyses can not be performed using the data presented here.

Longitudinal Analysis of IgG4 Production During Adalimumab Treatment

The IgG4 response is considered to be a delayed response against an antigen, since prolonged exposure to the antigen is required to reach high titers of IgG4 as for example shown for IgG4 against bee venom.

Here, we investigated the relative contribution of IgG4 to total IgG against adalimumab in time during 3 years of

treatment. To exclude drug interference that may hamper the analysis we studied this in nine patients in which adalimumab levels were undetectable for several consecutive time points (Fig. 2). Different patterns in the timing of IgG4 formation could be observed when analyzing longitudinal samples of these nine patients. In two patients the percentage of adalimumab specific IgG4 increased over time (Fig. 2a) as reported for different allergens [29]. In four patients the development of adalimumab specific IgG4 coincided with total IgG to adalimumab and in these patients the percentage of IgG4 was stable over time (Fig. 2b). In three patients the percentage IgG4 decreased in time (Fig. 2c).

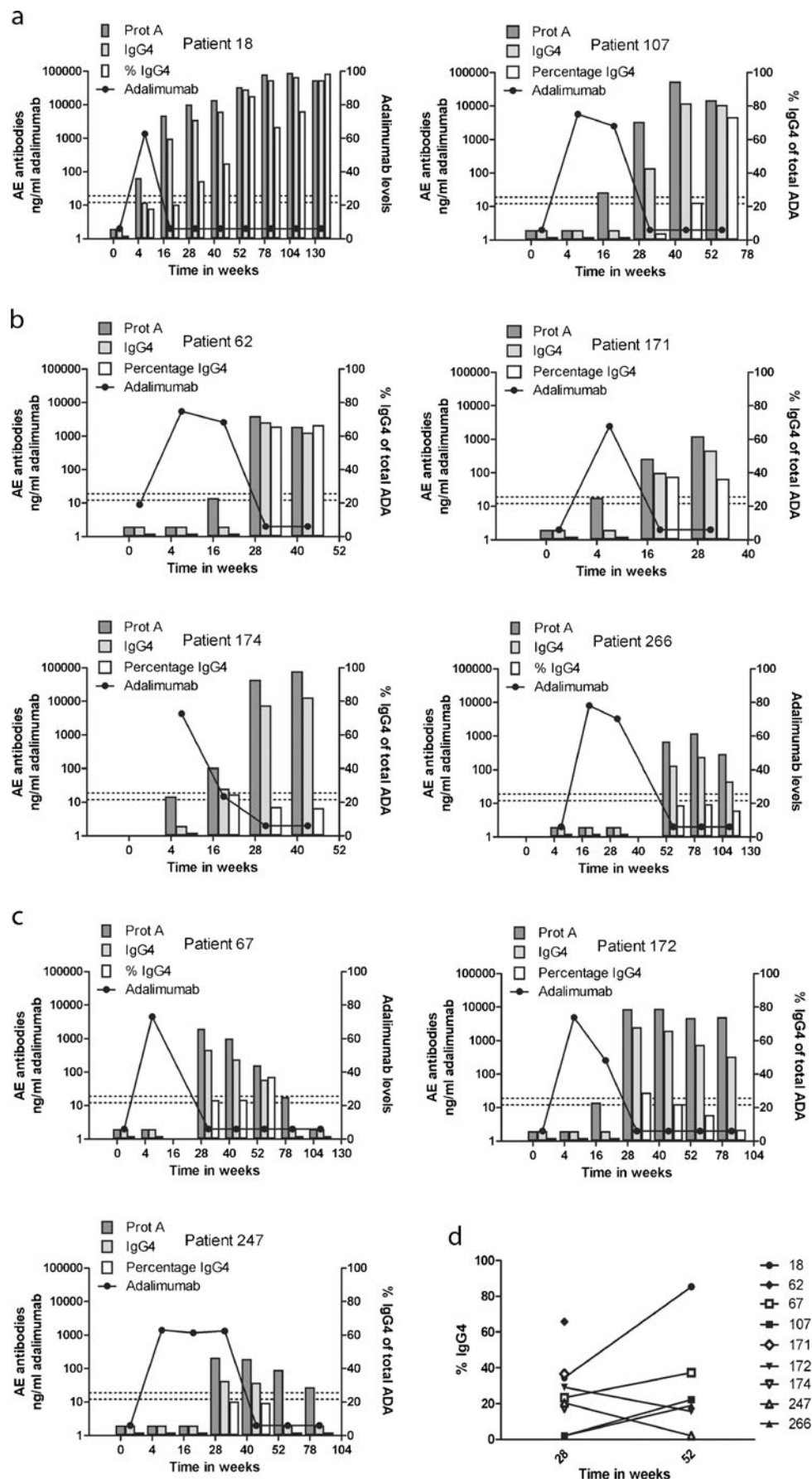
Figure 2d shows the percentage of adalimumab specific IgG4 after 28 and 56 weeks of treatment of the nine patients described above. We observed a wide variation in the relative contribution of IgG4 to total adalimumab specific IgG in the course of the IgG4 response. After 28 weeks, 0–66 % of total adalimumab specific IgG consisted of IgG4 and at 52 weeks the contribution of anti-adalimumab IgG4 was either increased or decreased, varying from 0 to 85 %. This variation in adalimumab specific IgG4 was not correlated with total IgG4 concentration in these sera (data not shown), suggesting that the variation in IgG4 ADA was not due to intrinsic differences within the patients leading to more or less IgG4 production in general.

The longitudinal analysis of anti-adalimumab IgG4 in relation to total IgG to adalimumab showed that there is also variation in the timing of IgG4 production against adalimumab. Some of the patients test positive for IgG4 after 16 weeks of treatment with adalimumab, while others only start producing detectable amounts of IgG4 after 52 weeks of treatment. The observation that the proportion of IgG4 antibodies may diminish in time is somewhat unusual and differs from what has been described previously for various allergens [1, 29].

Although it is not completely known yet what factors induce class switching to IgG4, it can be speculated that the immunological context of the patients and/or intensity of treatment (dosing and frequency) may play a role in class switching of anti-adalimumab antibodies to IgG4. Therefore, one of the explanations of the difference in IgG4 response between adalimumab treated patients and allergic patients might be that patients on adalimumab treatment receive adalimumab 40 mg every 2 weeks, while patients receiving allergen specific immunotherapy for allergy against for example grass pollen allergies receive 20 µg of allergen twice a week.

Together these data indicate that a large part of the ADA producing patients produce IgG4 against adalimumab. The timing at which patients start to produce as well as the percentage of IgG4 produced varies widely between patients. Also the course of the IgG4 response varies substantially between patients.

Fig. 2 Longitudinal analysis of total ADA and IgG4 ADA against adalimumab. Long term total ADA, IgG4 ADA and adalimumab measurements in nine patients with undetectable adalimumab levels at multiple consecutive time points. The *solid line* indicates the cut off of prot A ABT (12 AU) and the *dashed line* indicates cut off of the IgG4 ABT (19 AU). **a** In two patients the percentage IgG4 increased in time **b** in four the percentage IgG4 was stable and in **c** three the percentage IgG4 decreased in time **(c)** **d** Percentage IgG4 of total adalimumab specific IgG after 28 and 52 weeks of treatments in all nine patients in which adalimumab levels were undetectable at several time points



Because of the limiting ability of IgG4 to trigger immunological effector functions and tendency to form small immune complexes, IgG4 antibodies are presumed to be harmless and even thought to have a regulatory or protective effect against complement induced damage [29]. We have previously shown, using the same patient cohort, that antibody formation against adalimumab is linked with lower levels of functional drug levels and diminished clinical response. Here we show that a large portion of these antibodies consists of IgG4, suggesting that in this case IgG4 is not simply an innocent bystander [18]. Even though IgG4 is unable to activate complement and has a low affinity for Fc receptors, these antibodies may still be able to compete with TNF α for the binding to adalimumab and thereby neutralizing the drug resulting in clinical non-response.

Competing financial interests Dr. MT Nurmohamed has received consultancy fees from Abbott, Roche, Pfizer, MSD, UCB, BMS and Wyeth, payment for lectures from Abbott, Roche and Pfizer. Theo Rispens received honoraria for lectures from Pfizer. Prof Aarden has received honoraria for lectures from Abbott, Roche, and Pfizer.

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