

## EXTENDED REPORT

# Interleukin 10 promoter microsatellite polymorphisms are associated with response to long term treatment with etanercept in patients with rheumatoid arthritis

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**Objectives:** To analyse the association of interleukin 10 (IL10) promoter polymorphisms, which have been shown to be related to IL10 secretion capacity, with the response to long term treatment with etanercept in patients with rheumatoid arthritis (RA).

**Methods:** Fifty patients with active RA were treated for up to 4 years (median 39 months, range 3–52) with stable doses of etanercept as monotherapy. Treatment response was assessed as defined by the EULAR criteria in an intention to treat analysis, with the last observation carried forward. IL10 promoter microsatellite polymorphisms IL10.R and IL10.G were genotyped by fragment length analysis in patients and 189 healthy controls matched for ethnicity, age, and sex. Haplotypes were reconstructed using a method based on bayesian, coalescent theory with the PHASE software.

**Results:** IL10 microsatellite polymorphisms were not associated with susceptibility to RA. When patients with good treatment response ( $n=25$ ) were compared with patients with moderate ( $n=17$ ) or no response ( $n=8$ ), a significantly different distribution of the prevailing alleles R2, R3 and G9, G13, respectively, became evident. Good treatment response was associated with carriage of the R3 allele or R3-G9 haplotype, whereas the allele G13 and the haplotype R2-G13 predominated in patients with moderate or no response.

**Conclusion:** Genotyping of the IL10 promoter microsatellites may be useful in predicting the clinical response to etanercept in patients with RA. The high prevalence of the presumptive IL10 low producer allele R3 in patients with a favourable response suggests that IL10 promotes disease activity in RA under the specific condition of tumour necrosis factor antagonism.

Tumour necrosis factor  $\alpha$  (TNF $\alpha$ ) is a dominant regulator of the inflammatory cascade in rheumatoid arthritis (RA).<sup>1</sup> Today antagonism of TNF $\alpha$  is a well established therapeutic concept in RA.<sup>2–4</sup> However, not all patients respond to it equally well. Lack of efficacy in some patients may be due to the broad genetic heterogeneity of RA that results in a multitude of different phenotypes.<sup>5–6</sup> Synovial tissue cytokine production in patients with RA correlates with different patterns of lymphocyte infiltration, suggesting that several mechanisms can modulate the expression of the immune response in the synovial membrane.<sup>7</sup> Interleukin 10 (IL10) is a major immunoregulatory cytokine that is usually considered to mediate down regulation of the inflammatory response, as it inhibits the activation and effector functions of T cells, macrophages, and monocytes.<sup>8–9</sup> In particular, it acts as a negative autocrine regulator of TNF $\alpha$  and other proinflammatory cytokines.<sup>10</sup> Some other effects are not anti-inflammatory, as it stimulates B cell survival, proliferation, differentiation, and antibody isotype switching.<sup>9</sup> Raised levels of IL10 have been found in the serum and synovial fluid of patients with RA, possibly contributing to the diminished T cell function and increased antibody and rheumatoid factor production.<sup>11</sup> In fact, IL10 has been reported to activate B cells in vitro to promote autoantibody production like rheumatoid factor or antibodies against cyclic citrullinated peptide.<sup>12–14</sup>

About 75% of the variability in IL10 secretion is determined by genetic differences.<sup>15</sup> The IL10 gene maps to chromosome 1q31–q32.<sup>16</sup> In the IL10 promoter, multiple single nucleotide

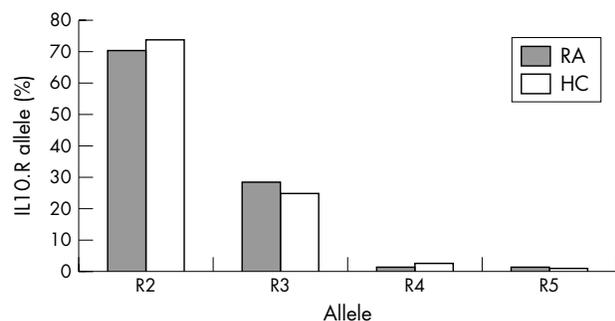
polymorphisms and two microsatellite polymorphisms have been identified. They combine to form four major haplotype families.<sup>17</sup> The CA dinucleotide repeat microsatellites, designated IL10.G and IL10.R, are located about 1.1 kb and 4.0 kb upstream of the transcription initiation site, respectively.<sup>18–19</sup> The IL10 secretion induced by lipopolysaccharide in vitro varies according to the haplotypic composition of the microsatellite alleles.<sup>20</sup> It has been suggested that susceptibility to RA is influenced by different IL10.R alleles.<sup>21</sup> More importantly, in patients with RA, associations between cytokine gene polymorphisms and the response to biological agents have also been described.<sup>22–24</sup> We suggested that genetically determined differences between patients in IL10 production may influence the treatment response to TNF blocking agents in RA. Thus, in this study we analysed, in patients with longstanding and active RA, the association of functionally relevant IL10 promoter microsatellite polymorphisms with the response to long term treatment with etanercept, a soluble TNF receptor fusion protein that binds and inactivates TNF.

## PATIENTS AND METHODS

### Study subjects

The study protocol was approved by the local independent ethics committee (Ethikkommission der Ärztekammer

**Abbreviations:** DAS28, 28 joint count Disease Activity Score; IL10, interleukin 10; PCR, polymerase chain reaction; RA, rheumatoid arthritis; TNF $\alpha$ , tumour necrosis factor  $\alpha$ .



**Figure 1** IL10.R allele distribution in patients with RA and healthy controls (HC). Bars represent the allele frequencies in the respective population. Allele distribution was not significantly different between patients with RA (n = 100 alleles) and healthy controls (n = 378 alleles).  $\chi^2$  (T4 statistics) = 0.62; p = 0.2232.

Westfalen-Lippe und der Medizinischen Fakultät der Westfälischen Wilhelms-Universität Münster). Before admission to the study, patients and controls gave written consent. All 50 patients were white German subjects and met the diagnostic criteria for RA as established by the American College of Rheumatology.<sup>25</sup> Thirty one patients were recruited from the outpatient Clinic for Rheumatology, Department of Medicine B, Münster University Hospital, Germany, and 19 from the Clinic of Rheumatology, University of Magdeburg, Vogelsang/Gommern, Germany. Thirty eight patients were female, 12 male. Their median age was 55 years (range 21–80). Thirty eight patients were positive for rheumatoid factor. Median disease duration at study admission was 6 years (range 1–39).

The patients had been pretreated either consecutively or simultaneously with a median of three disease modifying antirheumatic drugs (range 0–7). Forty one patients had received methotrexate, 28 sulfasalazine, 28 gold salts, 25 antimalarial drugs, 15 azathioprine, 12 leflunomide, 9 ciclosporin A, 3 cyclophosphamide, and, 1 patient each, infliximab, D-penicillamine, or interferon gamma. Healthy controls (n = 189) matched for ethnicity, age, and sex from the Prospective Cardiovascular Münster (PROCAM) study were included in the study.<sup>26</sup> Participants of this study were employees of Westphalian companies, and significant cardiovascular, pulmonary, metabolic, rheumatic, and renal disease

was excluded before study admission by review of the medical history and a physical examination.

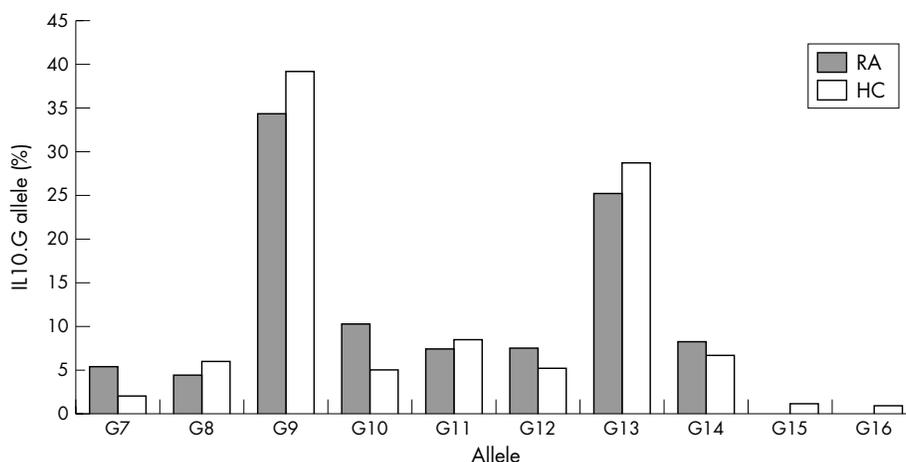
### Treatment

Recombinant human TNF receptor (p75)-Fc fusion protein (etanercept) was obtained from Wyeth-Ayerst Research, Münster, Germany. The patients received 25 mg etanercept as monotherapy twice a week subcutaneously for up to 4 years. Clinical evaluations comprised the calculation of a Disease Activity Score (DAS28)—a combined index based on a 28 tender and swollen joint count—erythrocyte sedimentation rate, and the patient's global disease activity measured on a visual analogue scale.<sup>27</sup> In Münster, follow ups were performed before the start of treatment and then every 3 months. In Magdeburg, patients were examined at baseline, after 3 and 6 months, and then every 6 months. Treatment response was assessed as defined by the EULAR criteria in an intention to treat analysis, with the last observation carried forward.<sup>28</sup> The EULAR response criteria are based on differences of the DAS28 at two different times and the current DAS28 reached under treatment. The median treatment period was 39 months (range 3–52), and only three patients were treated for less than 1 year. We thereby surveyed a total of 140 patient-years of etanercept use.

### Genotyping

DNA was extracted from EDTA anticoagulated blood of all 50 patients according to standard protocols.<sup>29</sup> The multiallelic IL10 microsatellites IL10.R and IL10.G were genotyped by fragment length analysis. Flanking primers were constructed: IL10.1, 5'-GTC.CTT.CCC.CAG.GTA.GAG.CAA.CAC.TCC-3' (5'-labelled with 6-FAM fluorescent dye, PE Applied Biosystems, Weiterstadt, Germany); IL10.2, 5'-CTC.CCA.AAG.AAG.CCT.TAG.TAG.TGT.TG-3'; IL10.3, 5'-CCC.TCC.AAA.ATC.TAT.TTG.CAT.AAG-3' (5'-labelled with HEX fluorescent dye, PE Applied Biosystems); and IL10.4, 5'-CTC.CG.CCA.GTA.AGT.TTC.ATC.AC-3'.

IL10.1 and IL10.2 amplified the IL10.G microsatellite, IL10.3 and IL10.4 the IL10.R microsatellite. A multiplex polymerase chain reaction (PCR) reaction was carried out in a thermal cycler (GeneAmp PCR System 9700, PE Applied Biosystems) under the following conditions: hot start at 94°C for 10 minutes, 30 cycles of 30 seconds at 95°C, 45 seconds at 65°C, 45 seconds at 72°C, final extension for 7 minutes at 72°C. The PCR reaction mixture contained 0.6 U AmpliTaq



**Figure 2** IL10.G allele distribution in patients with RA and healthy controls (HC). Bars represent the allele frequencies in the respective population. Allele distribution was not significantly different between patients with RA (n = 100 alleles) and healthy controls (n = 378 alleles).  $\chi^2$  (T4 statistics) = 8.09; p = 0.1684.

**Table 1** Linkage disequilibria between IL10.R and G alleles

IL10.R	Allele frequency	IL10.G	Allele frequency	Haplotype frequency	$\Delta$	$\chi^2$	p Value
R2	0.724	G7	0.025	0.019	0.001	0.02	0.9004
		G8	0.052	0.046	0.008	0.21	0.6533
		G9	0.379	0.151	-0.123	20.88	<0.0005
		G10	0.056	0.040	-0.001	0.01	0.9357
		G11	0.079	0.065	0.007	0.10	0.7576
		G12	0.052	0.052	0.014	0.79	0.3780
		G13	0.278	0.274	0.073	6.64	0.0100
		G14	0.067	0.067	0.018	1.26	0.2671
		G15	0.006	0.006	0.002	0.05	0.8250
		G16	0.004	0.004	0.001	0.08	0.7791
R3	0.253	G7	0.025	0.006	0.000	0.18	0.6784
		G8	0.052	0.004	-0.009	1.35	0.2508
		G9	0.379	0.207	0.111	22.05	<0.0005
		G10	0.056	0.017	0.002	0.01	0.9122
		G11	0.079	0.015	-0.005	0.12	0.7363
		G12	0.052	0.000	-0.013	4.40	0.0381
		G13	0.278	0.004	-0.066	27.40	<0.0005
		G14	0.067	0.000	-0.017	6.30	0.0131
		G15	0.006	0.000	-0.002	0.00	0.9661
		G16	0.004	0.000	-0.001	0.57	0.4544

Data represent the individual allele frequencies in the study group (n = 478), the haplotype frequencies as calculated with the PHASE software, differences  $\Delta$  between the expected and the observed haplotype frequencies, and levels of significance as determined by  $\chi^2$  test.

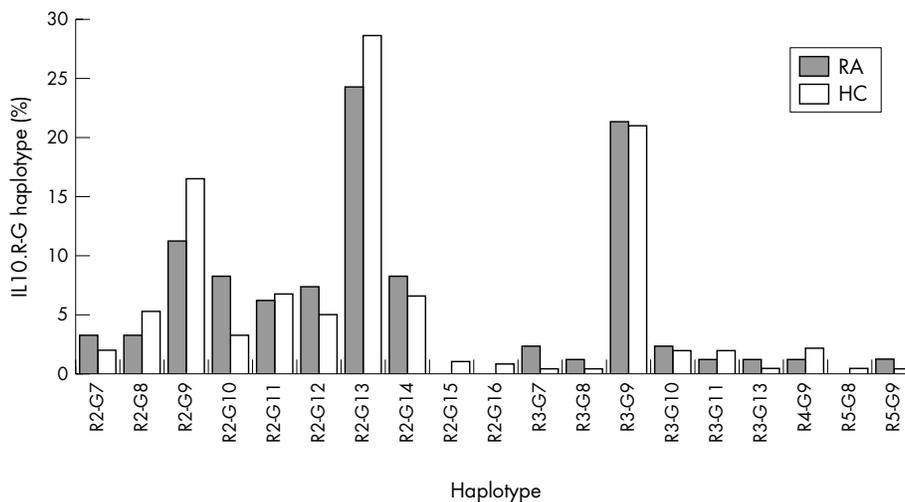
Gold, 0.2  $\mu$ M primers IL10.1, IL10.2, IL10.3, and IL10.4, 50  $\mu$ M dNTP, 1 mM MgCl<sub>2</sub>, and about 50 ng DNA in a total volume of 20  $\mu$ l. A DNA size standard (20  $\mu$ l of a 1:40 dilution of Genescan-ROX 500, PE Applied Biosystems) was added to 1  $\mu$ l of PCR product in a 96 well MicroAmp Optical Reaction Plate (PE Applied Biosystems). The samples were denatured after heating at 90°C for 2 minutes and then subjected to fragment length analysis on a four colour, laser induced fluorescence capillary electrophoresis system (ABI Prism 3700 Genetic Analyser, PE Applied Biosystems) using POP6 as polymer.

The size of microsatellite containing DNA fragments was measured by comparison with the DNA size standard using GeneScan Software. Analysis of the IL10.R microsatellite showed four alleles which ranged in size from 111 bp (IL10.R2) to 117 bp (IL10.R5). The 10 alleles of the IL10.G microsatellite ranged in size from 130 bp (IL10.G7) to 148 bp (IL10.G16). Each electrophoresis run included a negative

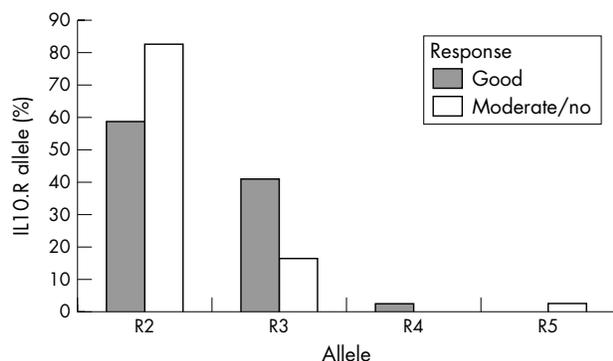
control and a DNA sample with known IL10.R and IL10.G genotypes as positive control. Specificity of genotyping was confirmed in selected cases (n = 20 chromosomes) by direct sequencing of PCR products (data not shown).

**Haplotype reconstruction and statistical analysis**

Haplotypes were reconstructed using a bayesian, coalescent theory based method with the PHASE software (version 2.0.2 for DOS).<sup>30 31</sup> The software incorporates a model that allows for recombination and decay of linkage disequilibrium with physical distance of alleles. Moreover, the type of polymorphism (single nucleotide or multiallelic, with/without stepwise mutation mechanism, respectively) is taken into account. Allele and haplotype frequencies in patients with RA and healthy controls as well as in subgroups of patients with different treatment response were compared by Monte Carlo simulation.<sup>32</sup> T4 statistics were applied to test for significance, which was assumed for a p value  $\leq 0.05$ . Phenotype



**Figure 3** IL10.R-G haplotype distribution in patients with RA and healthy controls (HC). Bars represent the haplotype frequencies in the respective population. Haplotype distribution was not significantly different between patients with RA (n = 100 alleles) and healthy controls (n = 378 alleles).  $\chi^2$  (T4 statistics) = 12.08; p = 0.2140.



**Figure 4** IL10.R allele distribution in patients with RA as related to etanercept response. Bars represent the allele frequencies in the respective patient subgroup. Allele distribution was significantly different between patients responding well to etanercept treatment (n = 50 alleles) and patients responding moderately or not at all (n = 50 alleles).  $\chi^2$  (T4 statistics) = 8.21;  $p = 0.0101$ .

frequencies were compared using  $\chi^2$  analysis or Fisher's exact test where appropriate. The strength of association was calculated as the odds ratio (OR) and is presented with 95% confidence intervals (CI). For calculation of linkage disequilibrium  $\Delta$  expected haplotype frequencies were estimated by multiplication of allele frequencies and subtracted from observed haplotype frequencies.  $\chi^2$  Analysis was used to determine the significance of the deviation from 0 to the  $\Delta$  value. Differences between quantitative data over time were analysed with the Wilcoxon's signed rank test. All statistical calculations were performed on a personal computer using the CLUMP (version 1.6 for DOS) or the MedCalc software (version 4.20.006 for Windows 95/NT).

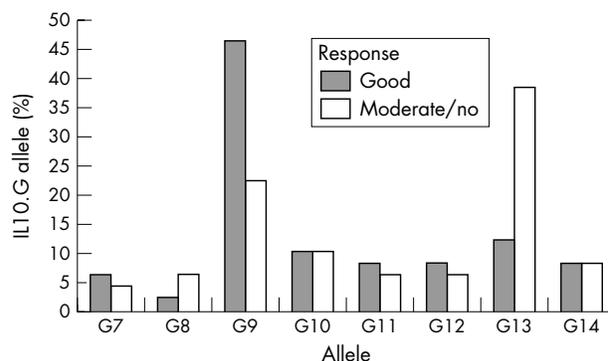
## RESULTS

### Clinical outcome

Within the first year of etanercept treatment, the median DAS28 was reduced from 6.2 (range 4.1–8.6) to 3.8 (1.2–8.6), the median glucocorticosteroid dosage from 7.5 mg (0–23) prednisolone equivalent to 5 mg (0–12.5), both highly significant at  $p \leq 0.0001$ . This reduction was stable, as no further significant changes were registered thereafter. According to the DAS28 before the start of treatment 45 patients had highly active disease. At their most recent visit, 18 patients were in remission, 7 patients had low, and 8 patients had high disease activity. As defined by the EULAR criteria, 25 patients responded well, 17 patients moderately well, and for 8 patients treatment failed. Fourteen patients stopped etanercept treatment within the study period. Reasons for discontinuation were ineffectiveness as assessed by the patients (n = 7), severe adverse events (n = 2), long journey to the study centre (n = 2), scheduled surgery (n = 1), desire for pregnancy (n = 1), and non-compliance (n = 1). The severe adverse events that led to discontinuation of treatment were, each in one patient, a perforation of the sigmoid colon and thrombocytopenia.

### Genotype distribution in patients with RA and healthy controls

In our study group we found four different alleles for the IL10.R, and 10 different alleles for the IL10.G microsatellite, respectively. The most common alleles were IL10.R2 (70% in patients with RA v 73% in healthy controls), IL10.R3 (28% v 25%), IL10.G9 (34% v 39%), and IL10.G13 (25% v 29%). Figures 1 and 2 show that there were no significant differences in the allele distribution between patients with RA and healthy controls. Haplotype reconstruction with the



**Figure 5** IL10.G allele distribution in patients with RA as related to etanercept response. Bars represent the allele frequencies in the respective patient subgroup. Allele distribution was significantly different between patients responding well to etanercept treatment (n = 50 alleles) and patients responding moderately or not at all (n = 50 alleles).  $\chi^2$  (T4 statistics) = 10.93;  $p = 0.0346$ .

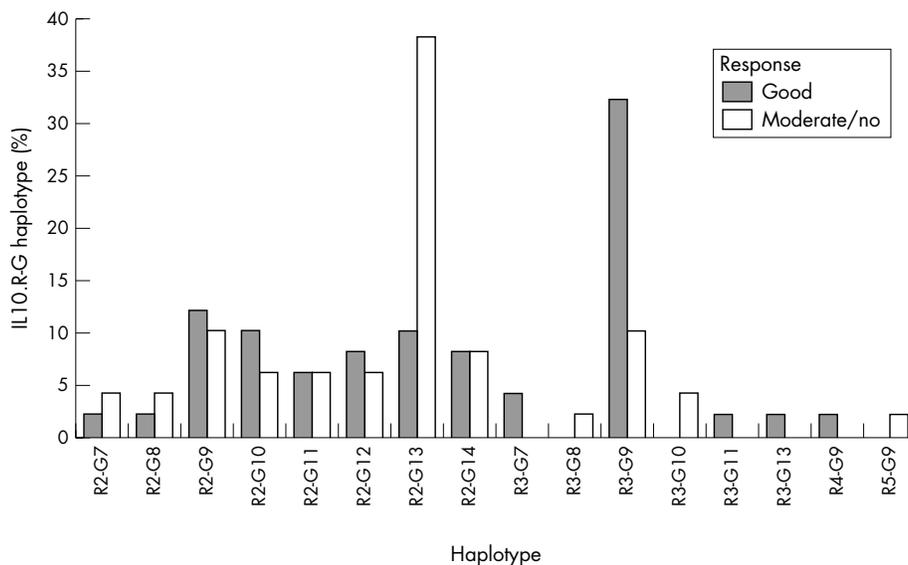
PHASE software resulted in a total of 19 different haplotypes with an average phase probability of 98%. Ninety five per cent of the reconstructed phases had a probability greater than 97%. The highest positive linkage was seen for R3-G9 and, less strong, for R2-G13, while R2-G9 and R3-G13 were negatively linked (table 1). The prevailing haplotypes were R2-G9 (11% in patients with RA v 16% in healthy controls), R2-G13 (24% v 28%), and R3-G9 (21% v 21%). Again, the haplotype distribution was not significantly different between patients and healthy controls (fig 3). However, the heterozygous presence of the R2-G10 haplotype was associated with a significant risk for RA with an odds ratio of 3.08 (95% CI 1.17 to 8.14). This haplotype was not found as a homozygous genotype. No other significant associations of individual alleles or haplotypes with RA susceptibility were detected.

### Genotype distribution and etanercept response

When patients with a good response to etanercept treatment as defined by the EULAR criteria were compared with patients with a moderate or no response, significant differences in the allele and haplotype distribution of the IL10 promoter microsatellite loci became evident (figs 4–6). The alleles R3 (40% in patients with good response v 16% in patients with moderate or no response), G9 (46% v 22%), and the haplotype R3-G9 (32% v 10%) prevailed in patients responding well, whereas the alleles R2 (58% v 82%), G13 (12% v 38%), and the haplotype R2-G13 (10% v 38%) were more common among patients with moderate or no response to the etanercept treatment. The carrier status of the IL10.R3 allele and the R3-G9 haplotype was thus associated with a good response to etanercept treatment, whereas the presence of the allele IL10.G13 or the haplotype R2-G13 resulted in odds ratios indicative of moderate or no response (table 2). The alleles IL10.R2 and IL10.G9 were associated with the treatment response without reaching significance. Comparison of the patients with moderate response with patients for whom treatment failed provided no further information as the latter group was too small to draw conclusions in the study of this highly polymorphic locus.

## DISCUSSION

Etanercept has been proved to have a sustained efficacy and an excellent safety profile when used for the treatment of patients with RA.<sup>2</sup> However, treatment with etanercept is expensive, and a causal relationship with the rare incidence of severe adverse events during the treatment cannot be definitively ruled out.<sup>33–39</sup> Thus, outcome predictors allowing



**Figure 6** IL10.R-G haplotype distribution in patients with RA as related to etanercept response. Bars represent the haplotype frequencies in the respective patient subgroup. Haplotype distribution was significantly different between patients responding well to etanercept treatment (n = 50 alleles) and patients responding moderately or not at all (n = 50 alleles).  $\chi^2$  (T4 statistics) = 17.83; p = 0.0186.

for individual treatment decisions are clearly needed. The well documented pleiotropic effects of IL10 in RA suggest that it is an important disease modifying factor, and gene polymorphisms associated with IL10 production capacity may, in consequence, function as prognostic markers.

Firstly we characterised our RA study group according to the IL10 promoter genotypes. In contrast with Eskdale *et al*, we found no associations of individual IL10.R or IL10.G alleles with disease susceptibility.<sup>21</sup> Our data corroborate results from a study performed in the UK with white and South African patients.<sup>40</sup> These discrepant findings may arise from considerable genetic heterogeneity between the different ethnic groups, but also from phenotypic variability of RA. Additionally, the IL10 microsatellites may be markers of disease susceptibility due to linkage disequilibrium rather than the primary causative genetic polymorphisms. For instance, the genotype of a single nucleotide polymorphism that is associated with high IL10 production has been found more frequently in patients with a higher rate of joint destruction and higher autoantibody titres.<sup>41</sup> Other, possibly not yet identified, polymorphisms in the IL10 promoter and their haplotypes may thus be more informative about susceptibility to RA.

The small number of patients for whom etanercept treatment failed in our study group raised the need to pool data from patients with moderate or no response into one group. In this way two equally sized groups were generated for comparison. The IL10 promoter microsatellite allele IL10.R3 and the haplotype R3-G9 prevailed significantly in

patients with a good response, whereas IL10.G13 and R2-G13 were more common among patients with a moderate or no response. These findings suggest that IL10 promoter microsatellite polymorphisms are predictors of etanercept response. The relevant alleles are common in all examined populations, thus enhancing the applicability of this genetic testing. Other than in previous studies, we did not investigate the association of genetic polymorphisms with the treatment response at a predefined point in time within the first months of treatment, but considered the most recent observation from clinical data collected over 4 years.<sup>22-24</sup> This approach may be more accurate as the response status varied to a considerable extent within the first year of treatment and stabilised thereafter.

The combination of polymorphic elements from the IL10 promoter in the form of haplotypes has previously been demonstrated.<sup>17-20</sup> In general, an analysis of haplotypes may be more informative when trying to find a functionally relevant sequence variation than the study of single microsatellite allele associations. We therefore reconstructed haplotypes using a method based on bayesian coalescent theory.<sup>30-31</sup> By this well established method the most common haplotypes were R2-G9, R2-G13, and R3-G9, matching previously published data of Eskdale *et al*, who deduced haplotypes by analysing family genotyping data.<sup>20</sup> Indeed, in contrast with the individual allele analysis, the haplotype analysis showed a significant association of the haplotype IL10.R2-G10 with susceptibility to RA. Admittedly, this association refers to a rare haplotype, and we do not believe

**Table 2** IL10 microsatellite phenotypes relative to etanercept response

Phenotype	Good response	Moderate/ no response	p Value	OR	95% CI
R2	21 v 4	24 v 1	0.3487	0.22	0.02 to 2.11
R3	17 v 8	7 v 18	0.0113	5.46	1.63 to 18.36
G9	16 v 9	11 v 14	0.2623	2.26	0.73 to 7.05
G13	6 v 19	16 v 9	0.0106	0.18	0.05 to 0.61
R2-G13	5 v 20	16 v 9	0.0046	0.14	0.04 to 0.50
R3-G9	14 v 11	5 v 20	0.0212	5.09	1.45 to 17.92

Data represent the number of phenotype positive v negative patients, relative to treatment response as defined by the EULAR criteria. The strength of association is quoted as the odds ratio (OR) with a 95% confidence interval (CI).

that it constitutes a major risk factor for RA. In the response analysis, consideration of the haplotypic organisation did not influence the strength of association observed. This is probably due to the strong positive linkage disequilibria between R2-G13 and R3-G9. However, these data support the suggestion that IL10 microsatellites are markers of treatment response due to linkage disequilibrium rather than the primary causative genetic polymorphisms.

Functional studies have shown that haplotypes containing the IL10.R3 allele are associated with a low lipopolysaccharide induced IL10 secretion in vitro.<sup>20</sup> If it is presumed that IL10 is a predominant anti-inflammatory cytokine, our finding that IL10.R3 is associated with a favourable response to etanercept is at a first glance surprising. However, in another study the allele of a single nucleotide polymorphism within the IL10 promoter that has been linked with low IL10 production capacity in vitro tended to be more prevalent among etanercept responders.<sup>22</sup> IL10 has both anti- and proinflammatory properties, and there is evidence that quantitative synovial IL10 mRNA correlates with joint damage progression.<sup>42</sup> It has been suggested that intrinsically high IL10 production is associated with severe, progressive RA.<sup>41</sup> In addition, abundant transcription of IL10 has been found in the synovium of patients with follicular synovitis who display higher serum TNF $\alpha$  levels than patients with diffuse synovitis.<sup>7, 43</sup> Thus, these patients with constitutively high IL10 production may represent a subset with more aggressive disease.

In summary, a good response to long term treatment with etanercept has been found to be associated with carriage of the IL10.R3 allele or the R3-G9 haplotype, whereas the allele IL10.G13 and the haplotype R2-G13 predominated in patients with moderate or no response. Our data suggest that genotyping of the IL10 promoter microsatellites may be useful in the prognostic estimation of the clinical response to etanercept in patients with RA. The high prevalence of the presumptive IL10 low producer allele R3 in patients with a favourable etanercept response suggests that IL10 promotes disease activity in RA under the specific condition of TNF antagonism.

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Competing interest statement: M Gaubitz is consultant for Wyeth-Ayerst Pharma, Germany, the manufacturer of etanercept, and for other producers of antirheumatic drugs.

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