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IL-6 pathway–driven investigation of response to IL-6 receptor inhibition in rheumatoid arthritis

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Clinical Trials Information

Registration names and numbers of tocilizumab clinical trials on Clinicaltrials.gov are OPTION NCT00106548, TOWARD NCT00106574, RADIATE NCT00106522, AMBITION NCT00109408, LITHE NCT00106535, and MEASURE NCT00535782.

ABSTRACT

Objectives To determine whether heterogeneity in interleukin-6 (IL-6), IL-6 receptor and other components of the IL-6 signalling pathway/network, at the gene, transcript and protein levels, correlates with disease activity in patients with rheumatoid arthritis (RA) and with clinical response to tocilizumab.

Design Biomarker samples and clinical data for five phase 3 trials of tocilizumab were analysed using serum, genotype and transcript analyses. Linear regression was then used to assess the association between these markers and either baseline disease activity or treatment response.

Results Higher baseline serum IL-6 levels were significantly associated (P<0.0001) with higher baseline DAS28, ESR, CRP and HAQ in patients with inadequate responses to disease-modifying anti-rheumatic drugs (DMARD-IR), patients with inadequate responses to anti-tumour necrosis factor (aTNF-IR) and patients who were naive/responders to MTX. Higher baseline serum IL-6 levels were also significantly associated with better clinical response to tocilizumab (versus placebo) as measured by cDAS28 in the pooled DMARD-IR (P<0.0001) and MTX-naive populations (P=0.04). However, the association with treatment response was weak. A threefold difference in baseline IL-6 level corresponded to only a 0.17-unit difference in DAS28 at week 16. IL-6 pathway SNPs and RNA levels also were not strongly associated with treatment response.

Conclusions Our analyses illustrate that the biological activity of a disease-associated molecular pathway may impact the benefit of a therapy targeting that pathway. However, the variation in pathway activity, as measured in blood, may not be a strong predictor.

These data suggest that the major contribution to variability in clinical responsiveness to therapeutics in RA remains unknown.

Trial registration Clinicaltrials.gov OPTION NCT00106548, TOWARD NCT00106574, RADIATE NCT00106522, AMBITION NCT00109408, LITHE NCT00106535, and MEASURE NCT00535782

Article Summary

Article focus

- This study aimed to determine whether genetic polymorphism and heterogeneity in expression of IL-6 pathway/network components could explain the variability in baseline disease activity and clinical response to tocilizumab in patients with RA

Key messages

- Higher baseline serum IL-6 levels were significantly associated with higher baseline disease activity (ESR, CRP, DAS28 and HAQ scores) in large populations of methotrexate-naive, DMARD-IR and aTNF-IR patients with RA. Higher baseline serum IL-6 levels also showed significant correlation with better clinical response to tocilizumab (cDAS28) in DMARD-IR and MTX-naive RA patients; however, the effect size was small, limiting the clinical usefulness of the marker in predicting treatment benefit. A threefold higher baseline IL-6 concentration corresponded to only an additional 0.17-unit decrease in DAS28 score in the DMARD-IR patients treated with tocilizumab
- None of the genetic polymorphisms in IL-6 or IL-6R showed any association with treatment response to tocilizumab
- No strong predictor of tocilizumab treatment response was identified; thus, the major factors underlying the patient-to-patient variability in clinical responsiveness to RA therapies remains unknown. This study illustrated well the

difficulty of identifying clinically useful predictive biomarkers in a complex chronic disease

Strengths and limitations of this study

- This study included well-characterised RA patients from five large phase 3 controlled clinical trials. Biomarker data were available for more than 3,700 patients. The extensive collection of RNA, DNA and serum samples allowed thorough investigation of the target-related heterogeneity
- This is the first demonstration of a significant association between baseline serum IL-6 levels and baseline disease activity in a large population of patients with RA, extending earlier findings of elevated serum IL-6 levels in RA patients compared with healthy controls
- The current biomarker analysis is focused on clinical usefulness and included only blood samples. Any potentially useful information about local regulation of IL-6 pathway gene transcription and protein level in tissues involved in RA could not be obtained

INTRODUCTION

Interleukin-6 (IL-6) is a pleiotropic cytokine important in autoimmune diseases, including rheumatoid arthritis (RA).[1] High concentrations of IL-6 are found in the synovial fluid and sera of patients with RA. IL-6 involvement in RA pathogenesis includes involvement in B-cell proliferation and cytokine production, matrix metalloproteinase expression, acute-phase response and anaemia.[2]

IL-6 signal transduction occurs through either a classical (*cis*-) or a *trans*-signalling pathway. In *cis*-signalling, IL-6 binds to membrane-bound IL-6 receptor alpha (mIL-6Rα) before forming a trimer with gp130. A signalling complex is formed when this heterotrimer forms a dimer with another IL-6/mIL-6R/gp130 complex.[1] However, though gp130 is ubiquitously expressed, mIL-6R expression is restricted to hepatocytes and a subset of leukocytes. The *trans*-signalling pathway makes use of a soluble form of IL-6R (sIL-6R), to which IL-6 binds before forming a heterodimer with membrane-bound gp130, allowing cells that do not express mIL-6R to be activated by IL-6.[1]

Studies of tocilizumab,[3-8] a humanised monoclonal antibody targeted to IL-6R (membrane and soluble) that inhibits the interaction of IL-6 with its receptor, have shown meaningful amelioration of RA. However, the response is heterogeneous, consistent with the responses of other RA therapies. In a pooled analysis of patients who were inadequate responders to disease-modifying anti-rheumatic drug (DMARD-IR) in pivotal trials, 39% of those receiving 8 mg/kg tocilizumab for 24 weeks failed to achieve American College of Rheumatology (ACR) 20 response (a 20% improvement), whereas 18% did achieve

ACR70 response (a 70% improvement). Therefore, there is great interest in discovering biomarkers to aid in physician decision-making by the accurate prediction of clinical response in individual patients.

IL-6 and IL-6R levels vary in the sera of RA patients.[9,10] It has been suggested that some of this heterogeneity is genetically determined. The rs8192284 A/C polymorphism is found at the cleavage site of *mIL-6R* (Gln 357/Asp358) and has been associated with increased sIL-6R levels and RA susceptibility,[11,12] whereas a polymorphism at –174 in the promoter region of *IL-6* (rs1800795) affects transcription activity and, thus, serum levels of IL-6.[13] Beyond RA, IL-6R polymorphisms that reduce cardiovascular risk[14,15] attenuate classical IL-6 signalling by increasing the cleavage rate of mIL-6R (rs2228145 and rs8192284) and mirror the effects of tocilizumab on levels of IL-6 (increase), C-reactive protein (CRP) and fibrinogen (decrease). In asthma, the rs4129267 polymorphism in IL-6R was found to be associated with increased risk, and Ferreira et al[16] suggested that an IL-6R antagonist may show efficacy in asthma in a genotype-dependent manner. The aim of this study was to determine whether baseline heterogeneity in IL-6, IL-6R and other components of the IL-6 signalling pathway/network, at the gene, transcript and protein levels, correlates with disease activity in RA and clinical response to tocilizumab.

METHODS

Patients and samples

Serum specimens used in this study were collected from five pivotal phase 3 studies of tocilizumab in patients with RA: RADIATE,[4] OPTION,[3] TOWARD,[5] AMBITION[6] and LITHE[7] (Table 1). DNA samples were from RADIATE, OPTION and TOWARD, and RNA samples were from RADIATE. These trials evaluated tocilizumab (4 mg/kg or 8 mg/kg), administered every 4 weeks over a period of 24 weeks or longer, compared with placebo. The study populations differed according to background therapy with methotrexate (MTX) (OPTION, LITHE, RADIATE), any DMARD (TOWARD) or no DMARD (AMBITION). They also differed according to previous inadequate response to anti-tumour necrosis factor (aTNF) agents (RADIATE), MTX (OPTION, TOWARD, LITHE) or neither (AMBITION). The study protocols were approved by relevant institutional review boards or ethics committees, and written informed consent was obtained from each patient. The studies were carried out in full concordance with the principles of the Declaration of Helsinki and with the laws and regulations of the country in which the research was conducted. DNA and RNA samples were collected only from patients who gave separate informed consent.

Serum analysis for IL-6 and sIL-6R

Three assays for serum IL-6 levels were used to analyse samples from TOWARD, as follows: Quantikine Human IL-6 Immunoassay (R&D Systems Inc., Minneapolis, MN), performed by Huntington Life Sciences (Huntington, UK) according to the instructions of the manufacturer (Roche Diagnostics, Penzberg, Germany) of the IL-6 assay[17]; IMPACT

platform,[18] performed by Roche Diagnostics; and RBM IL-6 assay, performed by Myriad RBM (Austin, TX). Given the tight correlations among these three assays (Supplementary Figure 1A), we chose to analyse data from the Quantikine Human IL-6 Immunoassay (R&D Systems Inc.) because they were available for nearly all patients in all five core studies. The limit of detection of this IL-6 assay is 3.12 pg/ml. The assay is unaffected by the addition of recombinant human sIL-6R[19]; therefore, it can be assumed that the data reflect the total concentration of IL-6 (i.e. the concentration of free IL-6 plus the amount of IL-6 bound to sIL-6R).

Serum concentrations of endogenous sIL-6R were measured using the Quantikine Human sIL-6R Immunoassay (R&D Systems Inc.) by Huntington Life Sciences according to the manufacturer's instructions. The sensitivity of the sIL-6R assay is 31.2 pg/ml. Measured sIL-6R concentrations are insensitive to the addition of recombinant human IL-6 but are sensitive to the presence of tocilizumab.[20] Therefore, it can be assumed that the sIL-6R data presented reflect the total amount of free sIL-6R plus sIL-6R complexed with IL-6 plus (in samples that contain tocilizumab) an unquantified fraction of sIL-6R bound by tocilizumab.

Genotyping

DNA samples were genotyped using the HumanHap550k BeadChip version 3.0 (Illumina, San Diego, CA) and custom chips using the OPA genotyping assay. To reduce the multiplicity of hypothesis testing, a targeted approach was taken. Three groups of genes were considered in this analysis. Tier 1 markers consisted of 26 polymorphisms

within the *IL-6* and *IL-6R* regions (Supplementary Table 1). The association of these markers with treatment benefit represents primary hypotheses. Tier 2 markers consisted of 233 polymorphisms within 22 genes identified as in the IL-6 pathway, termed the IL-6 canonical pathway (Supplementary Material). Tier 3 markers consisted of 1,005 polymorphisms in 67 genes, identified with GeneGo (Carlsbad, CA) software, related to IL-6 signalling, herein termed the IL-6 network (Supplementary Material). Markers for tiers 2 and 3 correspond to the decreasing strength of the hypothesis and the increasing dimensions compared with tier 1 markers. This approach provides greater power in the confirmatory analysis for the primary biomarker hypothesis yet allows for focused exploratory analysis to address the secondary hypotheses.

Transcript analysis

Two hundred thirty-three RNA samples, prepared from whole blood, at baseline were analysed using the GeneChip® Human Genome U133 Plus 2.0 Array (Affymetrix, Santa Clara, CA). After samples that failed quality control tests were removed (Supplementary Material), data for the remaining 217 samples were normalised using the Robust Multi-Array Analysis normalisation[21] (Bioconductor). Transcripts for *IL-6*, *IL-6R*, IL-6 signal transducer (*IL-6ST*) and *gp130* were extracted from the normalised data. IL-6 transcript levels were not analysed because of the low levels of signal observed. Three probe sets targeting *IL-6R* were analysed. Of those, 205945_at and 217489_at expression levels were found to be strongly correlated (Pearson's r^2=0.75), and the third probe set, 226333_at, had a weaker correlation with the other two (Pearson's r^2=0.34 and r^2=0.38).

These three probe sets represent the primary hypothesis in RNA transcripts. In addition, seven probe sets from *IL-6ST* were analysed as secondary markers.

Measures of disease activity and clinical response

Six different measures were used for disease activity at baseline: erythrocyte sedimentation rate (ESR), CRP, swollen joint count at 28 joints (SJC28), tender joint count at 28 joints (TJC28) and Health Assessment Questionnaire (HAQ), with disease activity at 28 joints (DAS28; derived from the four core components of ESR, SJC, TJC and patient global assessment) considered the primary end point. As a measurement of treatment response, change in DAS28 from baseline at week 16 (cDAS28) was mainly used. Unlike dichotomous responder criteria (e.g. ACR response, DAS28 remission and clinical disease activity index remission), this continuous measure captures the range of individual responses and is therefore more sensitive in detecting the effect of biomarkers across different levels of prevalence. In all studies except AMBITION, patients were allowed to enter escape therapy at 16 weeks if they had inadequate responses. Therefore, cDAS28 was used at week 16 to increase the sample size and to minimise bias.

Statistical analysis

Table 2 provides a summary of all markers analysed. Quality control analyses were performed for genotyping and gene expression data, as described in the Supplementary Material; assays and samples with poor data quality were removed. Distribution was assessed for each marker, and appropriate transformation was applied before further analysis. Minor allele frequencies and Hardy-Weinberg equilibrium were calculated for

single nucleotide polymorphisms (SNPs). Given that the distribution within each gene expression probe set was approximately normal, no further transformation was applied. Log transformation was applied to the protein markers IL-6 and sIL-6R because of the skewed distribution of raw values.

Correlations among the baseline markers were assessed within each sample type to enable understanding of the amount of information shared among the assays. For SNPs, linkage disequilibrium coefficients within IL-6 and IL-6R were obtained from HapMap22 based on the CEU (Utah residents of Northern and Western European ancestry from the CEPH collection) data. For the gene expression markers, correlation coefficients were calculated among probe sets within *IL-6R* and *IL-6ST*. For the proteins, correlation was calculated among the three assays for IL-6 and between IL-6 and sIL-6R. Pearson or Spearman rank correlation was used as appropriate.

Linear regression was used to assess the association between markers with baseline disease activity and markers with treatment response. Because of the skewed distribution of the raw values, log(ESR) and log(CRP+1) were used in the regression analysis. All patients were included in the regression analysis of baseline disease activity. In the regression analysis of cDAS28, separate analyses were undertaken in patients treated with tocilizumab and those administered placebo. Given that baseline DAS28 is strongly associated with change in DAS28 at week 16, it was included as a covariate in all models. No additional covariates were included in the model. In addition, the number of shared

epitope alleles[22] (0, 1 or 2) and tocilizumab dose were used as covariates in the analysis of genetic data.

The numbers of genetic markers tested were 18, 233 and 1,004, respectively, for the tier 1, 2 and 3 markers. In addition, six baseline end points were used on the baseline disease association analysis. Analyses were undertaken in the white population and the all-patient population. To adjust for multiple testing, the false discovery rate (FDR)[23] was calculated within each tier and within the white population and the all-patient population. This FDR accounted for the multiplicity of markers and the end points.

To help interpret the outcome of the regression analysis and to enable direct comparison of the analysis results across all markers, consistent representations of the results were used. For continuous markers, the regression coefficients were standardised to reflect how many units of change in the end points corresponded to a difference of 1 standard deviation (SD) in the marker value. For the SNP markers, an allelic model was used (i.e. two copies of the minor allele had twice the effect of one copy). The regression coefficient corresponded to the change in end point per copy of minor allele.

RESULTS

Associations with baseline disease activity

Higher baseline serum IL-6 levels were significantly associated with higher baseline DAS28, ESR, CRP and HAQ in all three populations analysed, with $P<0.0001$ in most cases (Table 3). The strongest association was observed between CRP and IL-6, for a

correlation coefficient of 0.29 to 0.36 in three populations (Supplementary Figure 1B). IL-6 was not strongly associated with SJC28 or TJC28. Baseline sIL-6R was not strongly associated with any baseline disease activity measures.

Among the baseline RNA transcripts, an association was observed between the IL-6R transcript and CRP and ESR at baseline (data not shown). No association was found with the other baseline disease activity measures. Association with CRP and ESR was also observed, to a lesser degree, for an IL-6ST transcript.

After correction for multiple testing, no significant associations were found for any of the *IL-6* or *IL-6R* polymorphisms genotyped with baseline disease activity. Of the 1,237 polymorphisms representing 22 genes in the IL-6 canonical pathway, 67 additional genes in the IL-6 network, six baseline disease activity end points and two patient populations (all-patient; white), six associations were found to have borderline significance after adjusting for multiple testing, with an FDR of approximately 5%. There were SNPs in *TOLLIP* and *RUNX1* versus ESR and SNPs in *IL1RAPL1* versus SJC and ESR. Such borderline significance meant the association should be treated as a weak hypothesis.

Associations with clinical response to tocilizumab

Higher baseline serum IL-6 concentrations were significantly associated with better clinical response to tocilizumab but not to placebo, as measured by cDAS28 in the pooled DMARD-IR population (P<0.0001) (Supplementary Table 2, Figure 1). The same effect was observed in the MTX-naive population (P=0.04). In the aTNF-IR population, the

association was not significant, partially because of the smaller sample size. In the DMARD-IR population, a threefold difference in baseline IL-6 concentration, equivalent to 1 SD in the baseline log(IL-6) distribution, corresponded to a small difference of 0.17 units in cDAS28 score at week 16. The association between serum IL-6 level and achievement of DAS28 remission (DAS28 <2.6) was analysed using receiver operating characteristic analysis in the pooled DMARD-IR population treated with tocilizumab (n=1,547). The area under the curve was only 0.59, suggesting that baseline serum IL-6 levels provided very little discrimination between those achieving and those not achieving DAS28 remission. In the placebo group, the direction of association was opposite that of tocilizumab-treated patients, indicating that baseline serum IL-6 level is not a general prognostic factor for clinical response in RA.

Baseline sIL-6R concentration in serum had no effect on treatment response in either the tocilizumab or the placebo group (Supplementary Table 2). IL-6R whole blood mRNA (in RADIATE) was not associated with clinical response to tocilizumab or placebo treatment (Supplementary Table 2). No *IL-6* or *IL-6R* polymorphisms were associated with cDAS28 after correction for multiple testing. Although rs2069840 and rs12700386 had a raw P<0.05 (Table 4), the estimated effect size was small. Each copy of the minor allele for rs2069840 corresponded to only a 0.3-unit difference in cDAS28. Of the SNPs in the canonical and the network or signalling pathway, rs973767 (an intronic SNP in *PTPN2*) had the lowest P value (5.7×10^{-5}) but was not significant after correction for multiple testing (FDR=0.057; Table 4).

DISCUSSION

We have investigated the associations among a number of DNA, RNA and protein biomarkers directly related to IL-6 signalling (Table 2) with baseline disease activity and treatment benefit from tocilizumab (Table 1). We demonstrate the modest association between serum IL-6 levels and clinical disease activity as measured by DAS28 and HAQ. Although serum IL-6 levels have previously been shown to be elevated in patients with RA compared with controls,[9,24] this is the first time a significant correlation between IL-6 level and disease activity has been demonstrated in a large population. IL-6 level accounted for only a small component of observed variance in disease activity (Supplementary Table 2, Figure 1). Of the four DAS28 components, serum IL-6 level was most strongly associated with ESR/CRP. This is not surprising given the pivotal role of IL-6 (and the pronounced inhibitory effect of tocilizumab) on the acute-phase response.[25] Interestingly, HAQ, a measure of patient-reported disability independent of inflammation, also had a significant association with serum IL-6 level.

Littman[26] proposes that IL-6/tocilizumab may represent 'low-hanging fruit' in terms of personalised health care in RA, but our analysis suggests otherwise. Higher baseline DAS28 is associated with higher change in DAS28 in response to therapy (Supplementary Figure 1C). When change in DAS28 is corrected for baseline DAS28, only a weak association with serum IL-6 is observed.

It is possible that discordance in IL-6 signalling activity between blood and synovial tissue provides an explanation for the lack of association with blood samples in our study.

However, this cannot explain the negative genetic data. The lack of genetic associations between polymorphisms in *IL-6R* (including those determining sIL-6R levels) and baseline disease activity[11,27] show that sIL-6R levels, though highly variable, are not appreciably different between patients with RA and those who are healthy.[28,29]

Our analyses illustrate that in a disease such as RA, variation in the abundance of a therapeutic target may not be a strong predictor of patient benefit. In particular, the response to tocilizumab is not dependent on IL-6R SNPs, which were found to be associated with asthma and cardiovascular risk. In addition, a genome-wide association approach using 1,600 patients from five controlled clinical trials also did not yield any strong predictor to tocilizumab treatment response.[30] These data suggest that the major contribution to variability in clinical responsiveness to therapeutics in RA remains unknown, and they illustrate the challenges of identifying predictive biomarkers, even in large, well-conducted studies.

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Competing interests

All authors are (or were at the time of the study) employees of Roche or Genentech, a member of the Roche group, and four authors (AH, AK, OH and RU) also own stock in Roche.

Author contributions

JW designed the study, conducted research, conducted the literature search, analysed and interpreted the data and drafted the manuscript; AP designed the study, conducted research, conducted the literature search, collected data, analysed and interpreted the data, oversaw data collection and drafted the manuscript ; RU analysed and interpreted the data and drafted the manuscript; SG designed the study and collected data; GL analysed and interpreted the data; CR analysed and interpreted the data; RB designed the study,

collected data and analysed the data; AK analysed and interpreted the data; AH designed the study and collected data; MM designed the study; OH interpreted the data and drafted the manuscript.

All authors revised the manuscript critically for important intellectual content and approved the final version for submission.

Ethics approval

The study protocols were approved by relevant institutional review boards or ethics committees, and written informed consent was obtained from each patient. The studies were carried out in full concordance with the principles of the Declaration of Helsinki and with the laws and regulations of the country in which the research was conducted. DNA and RNA samples were collected only from patients who gave separate informed consent.

Data sharing

No additional data are available at this time.

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TABLES

Table 1. Demographics of patients from whom biological samples were collected and analysed and whose data passed a quality control check

Sample type	DNA			RNA	Serum				
Study	**OPTION**	**RADIATE**	**TOWARD**	**RADIATE**	**OPTION**	**LITHE**	**TOWARD**	**AMBITION**	**RADIATE**
Patient population	MTX-IR	MTX-IR	DMARD-IR	aTNF-IR	MTX-IR	MTX-IR	DMARD-IR	MTX naive or free	aTNF-IR
Treatment	8 mg/kg TCZ + MTX	8 mg/kg TCZ + MTX	8 mg/kg TCZ + DMARD	8 mg/kg TCZ + MTX	8 mg/kg TCZ + MTX	8 mg/kg TCZ + MTX	8 mg/kg TCZ + DMARD	8 mg/kg TCZ + MTX	8 mg/kg TCZ + MTX
	4 mg/kg TCZ + MTX	4 mg/kg TCZ + MTX		4 mg/kg TCZ + MTX	4 mg/kg TCZ + MTX	4 mg/kg TCZ + MTX			4 mg/kg TCZ + MTX
	MTX	MTX	DMARD	MTX	MTX	MTX	DMARD	MTX	MTX
Total patients, n	286	178	463	217	603	1126	1010	581	431
TCZ, n	189	119	301	151	409	753	661	244	289
MTX, n	97	59	162	66	194	373	349	337	142
Age, years	51.3 (11.1)	51.5 (12.4)	54.2 (12.6)	53.2 (11.9)	51.0 (12.2)	51.9 (12.4)	53.4 (12.9)	50.3 (13.0)	52.9 (12.6)
Female, %	79.0	79.8	81.0	82.40	81.8	82.9	81.7	80.4	81.4
Height, cm	163 (9)	165 (8)	164 (9)	165 (8)	162 (9)	162 (9)	163 (9)	163 (9)	165 (8)
Weight, kg	73 (17)	77 (19)	76 (18)	77 (20)	79 (17)	73 (19)	74 (19)	73 (18)	75 (18)
RA duration, years	7.8 (7.3)	11.1 (8.3)	9.7 (9.1)	12.1 (9.6)	7.6 (7.3)	9.2 (8.0)	9.6 (8.8)	6.4 (8.2)	11.6 (9.1)
RF positive, %	76.2	79.8	75.6	76.5	77.6	82.1	77.1	72.5	75.2
White, %	73.8	93.3	76.2	Not applicable			Not applicable		
TCZ, n	141	109	233						
MTX, n	70	57	120						
DAS28	6.8 (0.9)	6.8 (1)	6.6 (1)	6.8 (0.9)	6.8 (0.9)	6.5 (0.9)	6.7 (1.0)	6.8 (0.9)	6.8 (1.0)
HAQ	1.6 (0.6)	1.7 (0.6)	1.5 (0.6)	1.7 (0.6)	1.6 (0.6)	1.5 (0.6)	1.5 (0.6)	1.5 (0.6)	1.7 (0.6)
SJC	20.9 (11.3)	18.8 (10.6)	20.5 (11.9)	18.1 (9.8)	20.0 (11.2)	16.9 (9.4)	19.6 (11.5)	19.8 (11.2)	18.9 (10.6)
TJC	32.3 (15.2)	31.2 (15.2)	30.1 (15.8)	31.4 (15.1)	32.6 (15.6)	28.3 (14.7)	30.0 (15.7)	31.9 (14.4)	31.1 (15.6)
CRP	2.4 (2.9)	3.7 (4.2)	2.3 (2.9)	3.3 (3.8)	2.6 (3.0)	2.2 (2.5)	2.6 (3.8)	3.0 (3.3)	3.2 (3.8)

CRP, C-reactive protein; DAS28 , Disease Activity Score using 28 joints; DMARD, disease-modifying antirheumatic drug; HAQ, Health Assessment Questionnaire; IR, inadequate responder; MTX, methotrexate; RA, rheumatoid arthritis; RF, rheumatoid factor; SJC, swollen joint count; TCZ, tocilizumab; TJC, tender joint count; aTNF, anti-tumour necrosis factor.

Values with numbers in parentheses are mean (SD).

Table 2. Summary of markers investigated. For the DNA markers, SNPs in IL-6 and IL-6R are considered tier 1, IL-6 network genes are considered tier 2, and IL-6 pathway genes are considered tier 3

Sample type	Target gene	Analytes, n	Assay (manufacturer)
Primary			
DNA	*IL-6*	10 SNPs	Bead-Chip arrays HumanHap550k version 3.0, OPA custom array (Illumina)
	IL-6R	18 SNPs	
RNA	*IL-6R*	3 probe sets	GeneChip Human Genome U133 Plus 2.0 Array (Affymetrix)
Serum	*IL-6*	1	Human IL-6 Immunoassay (Quantikine)
	sIL-6R	1	Human sIL-6R Immunoassay (Quantikine)
Secondary			
DNA	IL-6 network genes	233 SNPs (22 genes)	Bead-Chip arrays HumanHap550k version 3.0, OPA custom array (Illumina)
	IL-6 pathway genes	1004 SNPs (67 genes)	
RNA	*IL-6ST*	7 probe sets	GeneChip Human Genome U133 Plus 2.0 Array (Affymetrix)

IL-6, interleukin 6; IL-6R, IL-6 receptor; IL-6ST, IL-6 signal transducer; sIL-6R, soluble IL-6R; SNP, single-nucleotide polymorphism.

Table 3. Association of markers with baseline disease activity. All results for serum markers are shown. For RNA, the probe sets within each gene were strongly correlated. One probe set each in IL-6R and IL-6ST is shown. For DNA, only associations with raw P<0.01 (tier 1) and FDR P<0.05 (tiers 2 and 3) were shown. FDR was calculated within the population (white or all) and within the tier

Serum protein and RNA markers									
		Serum IL-6			Serum sIL-6R			IL-6R 217489_s_at	IL-6ST 234967_at
End point		AMBITION	RADIATE	Pooled DMARD-IR	AMBITION	RADIATE	Pooled DMARD-IR	RADIATE	RADIATE
DAS28	n	480	322	2286	577	423	2620	217	217
	β	0.209	0.257	0.201	–0.007	0.075	0.054	0.109	–0.04
	r^2	0.051	0.072	0.044	0	0.006	0.003	0.013	0.002
	p	<0.0001	<0.0001	<0.0001	0.8478	0.1018	0.0044	0.09	0.54
TJC	n	481	327	2305	580	428	2642	217	217
	β	–0.186	1.455	0.138	–0.154	1.28	0.054	0.27	1.276
	r^2	0	0.009	0	0	0.007	0	0	0.007
	p	0.7742	0.0938	0.6611	0.7967	0.0889	0.8558	0.79	0.2140
SJC	n	481	327	2305	580	428	2642	217	217
	β	0.247	1.421	0.813	–0.679	–-0.069	0.52	–0.864	0.218
	r^2	0	0.018	0.006	0.004	0	0.002	0.008	0
	p	0.6279	0.0158	0.0003	0.1424	0.8938	0.013	0.1950	0.7440
ESR	n	481	327	2299	580	428	2635	217	217
	β	0.19	0.172	0.151	0.039	–0.003	0.023	0.164	–0.132
	r^2	0.088	0.091	0.055	0.004	0	0.001	0.082	0.053

	p	<0.0001	<0.0001	<0.0001	0.132	0.9158	0.0672	<0.0001	0.0007
CRP	n	481	327	2305	580	428	2642	217	217
	β	0.363	0.429	0.35	0.007	0.019	0.019	0.228	–-0.145
	r^2	0.288	0.358	0.309	0	0.001	0.001	0.089	0.036
	p	<0.0001	<0.0001	<0.0001	0.8103	0.5935	0.1242	<0.0001	0.005
HAQ	n	480	324	2165	579	425	2465	217	217
	β	0.085	0.145	0.084	0.002	0.074	0.024	0.048	–0.103
	r^2	0.019	0.062	0.018	0	0.016	0.002	0.007	0.031
	p	0.0022	<0.0001	<0.0001	0.933	0.0089	0.0504	0.22	0.009

DNA markers

Tier	Gene	Chr	SNP	Population	MAF	End point	n	β	SE	Raw P	FDR P
1	*IL-6R*	1	rs1386821	White	0.17	HAQ	708	0.135	0.041	0.001	0.18
				All	0.15	HAQ	845	0.118	0.040	0.004	0.52
3	*TOLLIP*	11	rs5743899	All	0.22	ESR	893	0.15	0.03449	1.5E-05	0.046
	RUNX1	21	rs2252585	All	0.32	ESR	905	0.1209	0.02884	3.0E-05	0.046
	IL-1RAPL1	23	rs12559028	All	0.49	SJC	905	2.182	0.5349	4.9E-05	0.050
			rs5943618	All	0.44	SJC	905	2.27	0.5424	3.1E-05	0.046
			rs4829239	All	0.38	SJC	905	2.321	0.5592	3.6E-05	0.046
			rs5927671	All	0.45	ESR	897	0.1181	0.02853	3.8E-05	0.046

β, regression coefficient, representing the difference in the end point corresponding to standard deviation in marker value; Chr, chromosome; CRP, C-reactive protein; DAS28, Disease Activity Score using 28 joints; DMARD, disease-modifying antirheumatic drug; ESR, erythrocyte sedimentation rate; HAQ, Health Assessment Questionnaire; IL-6ST, IL-6 signal transducer; IR, inadequate responder; MAF, minor allele frequency; MTX, methotrexate; SJC, swollen joint count; TCZ, tocilizumab; TJC, tender joint count.

Table 4. Association of baseline markers with treatment response measured by change in DAS28 from baseline at week 16. Baseline DAS28 and TCZ dose were included in the model as covariates. FDR was calculated within the white or all population and within the tier

All association results for primary DNA markers											
Gene	SNP	All DMARD-IR, TCZ					White DMARD-IR, TCZ				
		MAF	n	β	SE	Raw P	MAF	n	β	SE	Raw P
IL-6	rs12700386	0.19	583	0.22	0.11	0.049	0.18	468	0.16	0.13	0.20
	rs2069833	0.35	584	–0.08	0.08	0.34	0.42	469	–0.13	0.09	0.18
	rs2069837	0.08	586	–0.11	0.15	0.44	0.08	471	–0.05	0.16	0.75
	rs2066992	0.12	586	–0.09	0.12	0.49	0.06	471	–0.11	0.21	0.61
	rs2069840	0.32	584	0.24	0.09	0.007	0.33	469	0.30	0.10	0.0026
	rs1554606	0.4	586	–0.10	0.08	0.23	0.45	471	–0.15	0.09	0.12
	rs2069845	0.4	583	–0.10	0.08	0.23	0.45	468	–0.15	0.09	0.12
	rs2069861	0.07	584	–0.10	0.15	0.53	0.08	469	–0.11	0.16	0.50
	rs10242595	0.36	586	–0.02	0.08	0.86	0.32	471	–0.02	0.10	0.80
	rs11766273	0.07	586	0.10	0.16	0.53	0.08	471	0.05	0.17	0.75
IL-6R	rs1386821	0.15	586	0.02	0.12	0.87	0.17	471	0.06	0.13	0.67
	rs4075015	0.39	584	–0.06	0.09	0.50	0.41	469	–0.07	0.10	0.46
	rs6684439	0.41	586	0.03	0.08	0.76	0.39	471	0.06	0.10	0.55
	rs4845618	0.43	582	0.03	0.08	0.74	0.44	467	0.00	0.09	0.99
	rs8192282	0.15	581	–0.09	0.12	0.46	0.16	468	–0.08	0.13	0.53
	rs7549250	0.43	575	0.07	0.08	0.43	0.44	462	0.02	0.09	0.81
	rs4553185	0.43	586	0.04	0.08	0.60	0.44	471	0.01	0.09	0.90
	rs4845623	0.44	586	–0.01	0.08	0.92	0.41	471	0.01	0.10	0.89

	rs4537545	0.44	586	–0.04	0.08	0.60	0.41	471	–0.01	0.10	0.93
	rs4129267	0.41	586	–0.02	0.08	0.77	0.4	471	0.02	0.10	0.87
	rs8192284	0.41	582	–0.03	0.08	0.76	0.4	467	0.02	0.10	0.87
	rs11265618	0.17	586	–0.05	0.11	0.64	0.17	471	–0.04	0.13	0.73
	rs4329505	0.16	582	–0.03	0.11	0.81	0.16	467	–0.02	0.13	0.87
	rs4240872	0.24	586	0.03	0.10	0.79	0.23	471	–0.01	0.11	0.93
	rs4509570	0.24	580	0.02	0.10	0.85	0.23	466	–0.02	0.11	0.83
	rs2229238	0.19	584	–0.03	0.11	0.75	0.19	469	0.00	0.12	0.98
	rs7514452	0.19	586	–0.03	0.11	0.77	0.19	471	0.00	0.12	0.98
	rs7526293	0.2	584	–0.04	0.10	0.71	0.2	469	–0.03	0.12	0.79

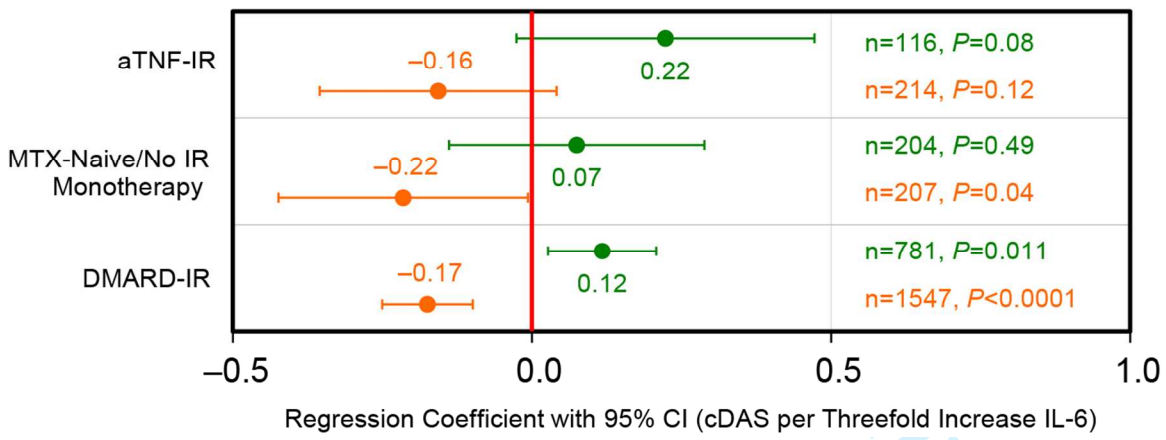
DNA markers analyzed in the pooled DMARD-IR population with an FDR* of <0.1

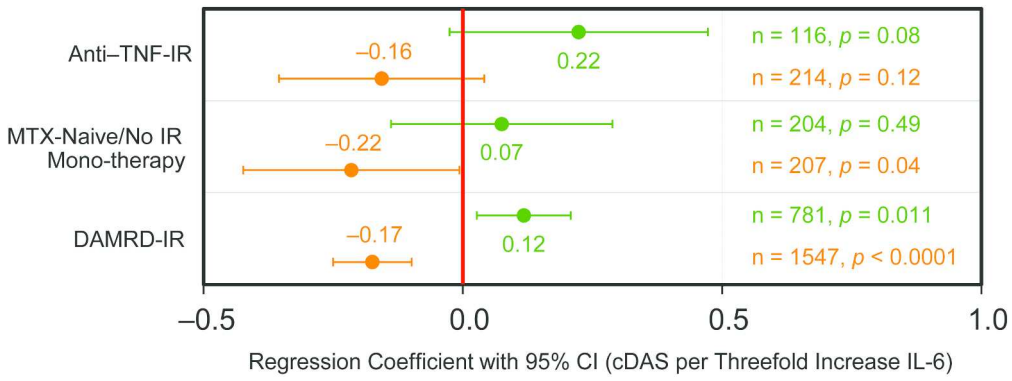
Tier	Gene	SNP	Population	MAF	n	β	SE	RAW P	FDR P
1	*IL-6*	rs2069840	White	0.33	469	0.30	0.10	0.0026	0.073
3	*PTPN2*	rs973767	All	0.14	586	0.48	0.12	7.2E-05	0.072
			White	0.16	471	0.53	0.13	5.7E-05	0.057

β, regression coefficient, representing the difference in DAS28 corresponding to each SD in marker value; DAS28, Disease Activity Score using 28 joints; DMARD, disease-modifying antirheumatic drug; FDR, false discovery rate; IL-6, interleukin-6; IL-6R, IL-6 receptor; IR, inadequate responder; MAF, minor allele frequency; SNP, single nucleotide polymorphism; TCZ , tocilizumab.

FIGURE

Figure 1. Serum IL-6 concentration association with response to TCZ. Forest plots show the effect and 95% CI for the association of IL-6 with cDAS28 at 16 weeks across treatment lines. cDAS28, change in DAS28 from baseline at week 16; CI, confidence interval; DMARD, disease-modifying antirheumatic drug; IR, inadequate responder; MTX, methotrexate; SD, standard deviation; TCZ, tocilizumab; TNF, tumour necrosis factor. •, MTX/DMARD; •, TCZ+MTX/DMARD.





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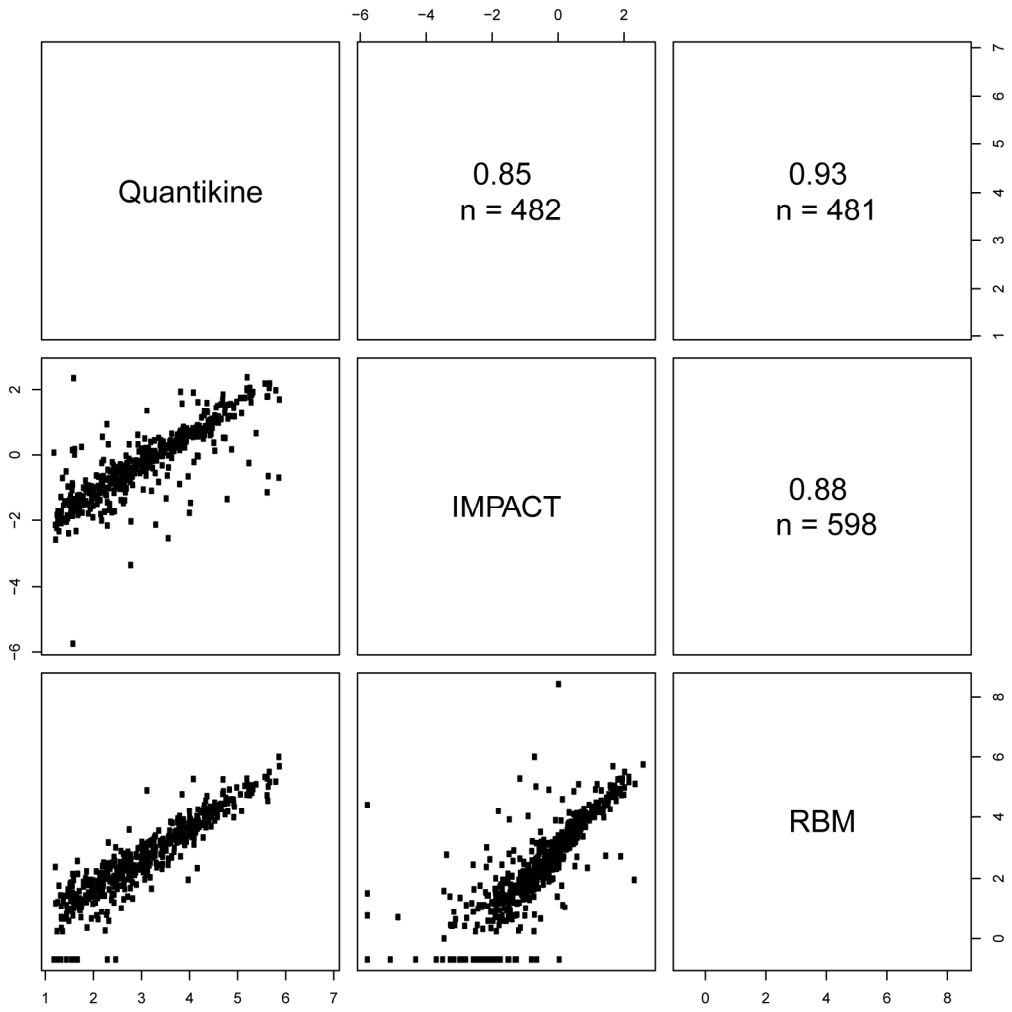

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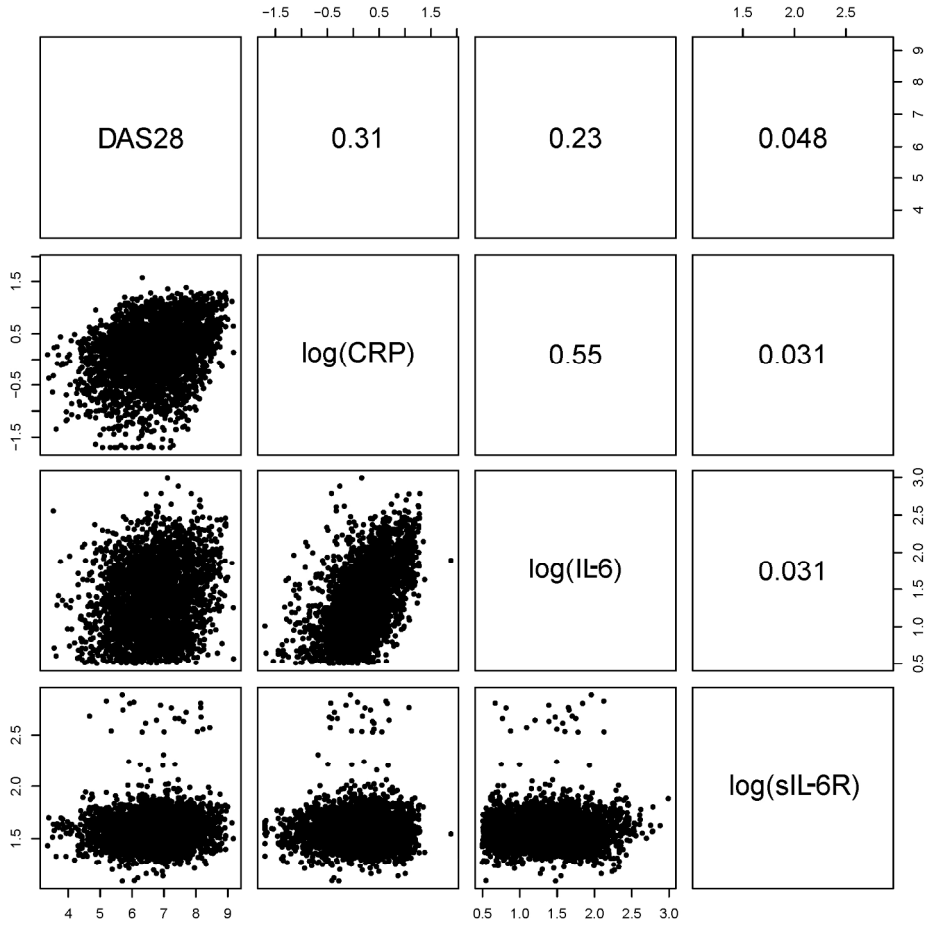

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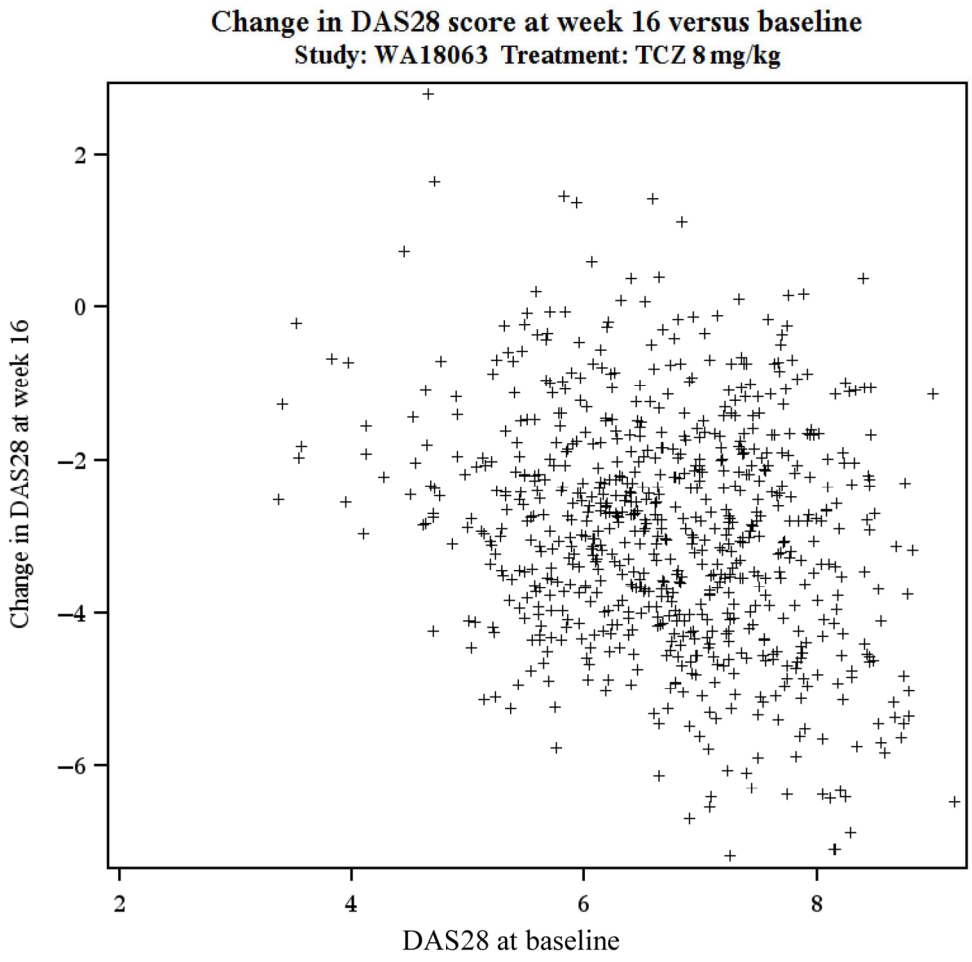


133x134mm (300 x 300 DPI)

SUPPLEMENTARY MATERIAL

SNPs investigated on IL-6 network–associated genes

Tier 2 SNPs include 233 polymorphisms within 22 genes: *ADAM10, ADAM17, CEBPB, CEBPD, IL6ST, JAK1, MAP2K1, MAP2K2, MAPK1, MAPK3, NFKB1, NFKB2, NR3C1, PTPN11, RAF1, REL, RELA, RELB, SOCS3, SOS1, SOS2,* and *STAT3*. Tier 3 SNPs include 1005 polymorphisms in 67 genes: *TNFRSF4, MTHFR, IL10, ATF3, IL1R2, IL1R1, IL1RL2, IL1RL1, IL1F9, IL1RN, STAT1, CTLA4, FN1, IRAK2, MYD88, CD86, IL1RAP, TLR1, RFC1, SPP1, TLR2, PIK3R1, IRF1, IL4, EGR1, TNF, MAPK14, NFKBIE, IRAK1BP1, MAP3K7, IFNGR1, ESR1, RAC1, TRIP6, IRF5, IKBKB, MYC, TLR4, CHUK, TOLLIP, TRAF6, RELT, IL10RA, TIRAP, STAT6, IRAK3, NFKBIA, SOCS1, UBB, NOS2, STAT5A, MAP3K14, TYMS, PTPN2, BCL2, ELAVL1, TYK2, PIK3R2, NFKBIB, BCL2L1, IL10RB, IFNGR2, RUNX1, IL1RAPL1, BTK, IRAK1,* and *IKBKG*. The polymorphism IDs are available from the corresponding author upon request.

Method for genotyping data QC

Before genotyping, a Y-chromosome test assay was performed for all samples. Sex results of this assay were compared with clinical data, and only samples with concordance in sex assignment were selected for genotyping. DNA samples genotyped using the Illumina standard arrays or custom arrays and with call rates <99% or <95%, respectively, were re-genotyped Re-assayed samples with call rates consistently below the threshold were removed from the analysis. Sample pairs with kinship coefficient ≥0.5 were excluded. Markers with call rates <95% were individually reviewed and re-clustered. The quality of markers with call rates ≥95% was assessed using Infinium genotyping QC metrics (Illumina). Single-nucleotide polymorphisms (SNPs) with either >5% missing data or with a minor allele frequency <1% were excluded from the analysis. Chi-square tests of Hardy-Weinberg equilibrium were conducted in white patients; the results were used, along with estimates of minor allele frequency, to assist in the interpretation of associations.

Method for gene expression data QC

Quality control metrics on MAS5 normalized data, box plots, MvA plots, and principal component analyses were performed using the Bioconductor QC method; 11 chips were identified as having poor quality. Five samples were further excluded because of missing Disease Activity Score using 28 joints (DAS28) erythrocyte sedimentation rate (ESR) data at baseline or week 16.

Supplementary Table 1.SNPs on IL-6 and IL-6R SNPs in the primary analysis and the pair-wise linkage disequilibrium coefficient (R^2) based on data from HapMap22.

Gene: IL6, Chromosome 7			Pair-wise R^2								
SNP ID	**Position**	**Function**	**rs1270038**	**rs2069833**	**rs2069837**	**rs2066992**	**rs2069840**	**rs1554606**	**rs2069845**	**rs2069861**	**rs10242595**
rs12700386	22,729,534	5' upstream									
rs2069833	22,734,189	Intronic	0.134								
rs2069837	22,734,552	Intronic	0.021	0.079							
rs2066992	22,734,774	Intronic	0.013	0.048	0.003						
rs2069840	22,735,097	Intronic	0.333	0.512	0.033	0.02					
rs1554606	22,735,232	Intronic	0.149	0.935	0.084	0.051	0.548				
rs2069845	22,736,674	Intronic	0.149	0.935	0.084	0.051	0.548	1			
rs2069861	22,738,179	3' downstream	0.021	0.065	0.005	0.003	0.033	0.06	0.06		
rs10242595	22,740,756	3' downstream		0.146	0.235	0.143	0.006	0.162	0.162	0.022	
rs11766273	22,742,188	3' downstream		0.11	0.009	0.005	0.056	0.103	0.103	0.009	0.037

Gene IL-6R, Chromosome 1			Pair-wise R^2															
SNP ID	**Position**	**Function**	**rs1386821**	**rs4075015**	**rs6684439**	**rs4845618**	**rs8192282**	**rs7549250**	**rs4553185**	**rs4845623**	**rs4537545**	**rs4129267**	**rs8192284**	**rs1126561**	**rs4329505**	**rs4240872**	**rs4509570**	**rs22292**
rs1386821	152,648,673	Intronic																
rs4075015	152,655,820	Intronic	0.169															
rs6684439	152,662,463	Intronic	0.012	0.04														
rs4845618	152,666,639	Intronic	0.003	0.028	0.033													
rs8192282	152,668,303	Intronic	0.008	0.011	0.013	0.219												
rs7549250	152,670,960	Intronic	0.001	0.013	0.029	0.905	0.198											
rs4553185	152,677,579	Intronic	0.001	0.013	0.029	0.905	0.198	1										
rs4845623	152,682,401	Intronic	0.009	0.048	0.07	0.411	0.085	0.504	0.504									
rs4537545	152,685,503	Intronic	0.003	0.042	0.073	0.393	0.082	0.486	0.486	0.964								

rs4129267	152,692,888	Intronic	0.003	0.042	0.073	0.393	0.082	0.486	0.486	0.964	1							
rs8192284	152,693,594	Intronic	0.002	0.034	0.07	0.368	0.085	0.455	0.455	0.928	0.964	0.964						
rs11265618	152,696,716	Intronic	0.005	0.011	0.012	0.194	0.887	0.176	0.176	0.101	0.098	0.098	0.101					
rs4329505	152,699,044	Intronic	0.005	0.011	0.012	0.194	0.887	0.176	0.176	0.101	0.098	0.098	0.101	1				
rs4240872	152,702,819	Intronic	0.043	0.095	0.009	0.316	0.071	0.356	0.356	0.18	0.205	0.205	0.18	0.087	0.087			
rs4509570	152,703,008	Intronic	0.043	0.095	0.009	0.316	0.071	0.356	0.356	0.18	0.205	0.205	0.18	0.087	0.087	1		
rs2229238	152,704,520	3' UTR	0.026	0.034	0.004	0.254	0.074	0.285	0.285	0.125	0.148	0.148	0.155	0.066	0.066	0.752	0.752	
rs7514452	152,704,708	3' UTR	0.028	0.027	0.005	0.23	0.077	0.26	0.26	0.105	0.126	0.126	0.132	0.068	0.068	0.715	0.715	0.9
rs7526293	152,710,833	3' downstream	0.022	0.021	0.003	0.228	0.068	0.256	0.256	0.111	0.134	0.134	0.139	0.06	0.06	0.688	0.688	0.9

IL-6=interleukin-6; IL-6R=IL-6 receptor; SNP=single nucleotide polymorphism.

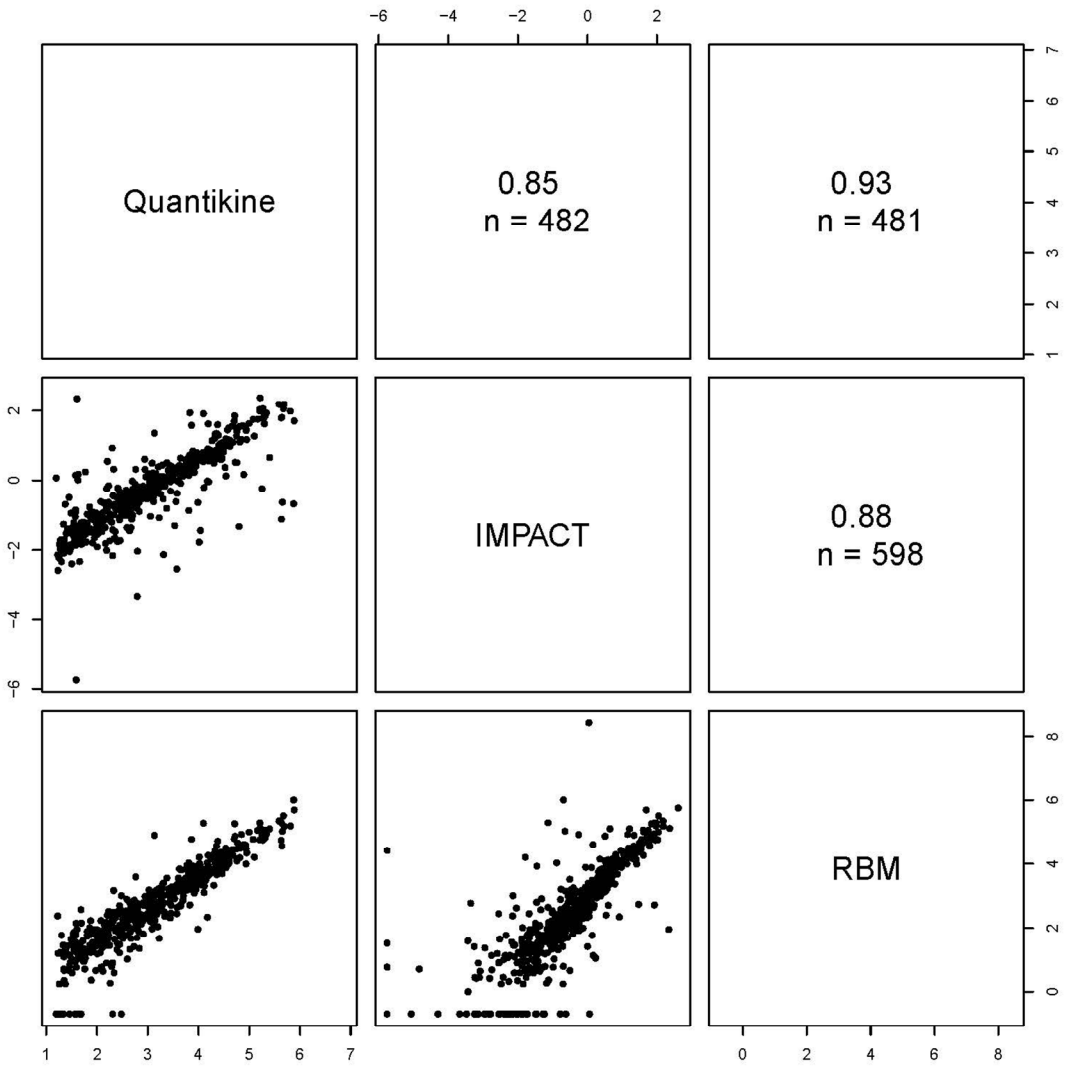
Supplementary Table 2. Association of baseline markers with treatment response measured by change in DAS28 from baseline at week 16.

Serum protein markers							
		Pooled DMARD-IR		**AMBITION**		**RADIATE**	
		TCZ	**Placebo**	**TCZ**	**Placebo**	**TCZ**	**Placebo**
IL-6	n	1547	781	207	204	214	116
	β	–0.17	0.12	–0.22	0.07	–0.16	0.22
	SE	0.04	0.05	0.11	0.11	0.10	0.13
	p	<0.0001	0.011	0.04	0.49	0.12	0.08
sIL-6R	n	1772	897	247	250	287	144
	β	0.04	0.01	0.03	–0.01	0.11	–0.04
	SE	0.03	0.05	0.13	0.14	0.11	0.14
	p	0.19	0.77	0.80	0.97	0.32	0.76

Primary RNA markers					
			RADIATE		
Gene	**Probe set**		**TCZ 8 mg/kg**	**TCZ 4 mg/kg**	**Placebo**
IL-6R	205945_at	n	76	75	66
		β	–0.1	0.06	–0.03
		SE	0.17	0.15	0.17
		p	0.53	0.68	0.86
	217489_s_at	n	76	75	66
		β	–0.14	0.06	0.09
		SE	0.19	0.14	0.14
		p	0.47	0.68	0.53
	226333_at	n	76	75	66
		β	0.03	0.11	0.28
		SE	0.19	0.12	0.17
		p	0.87	0.39	0.12

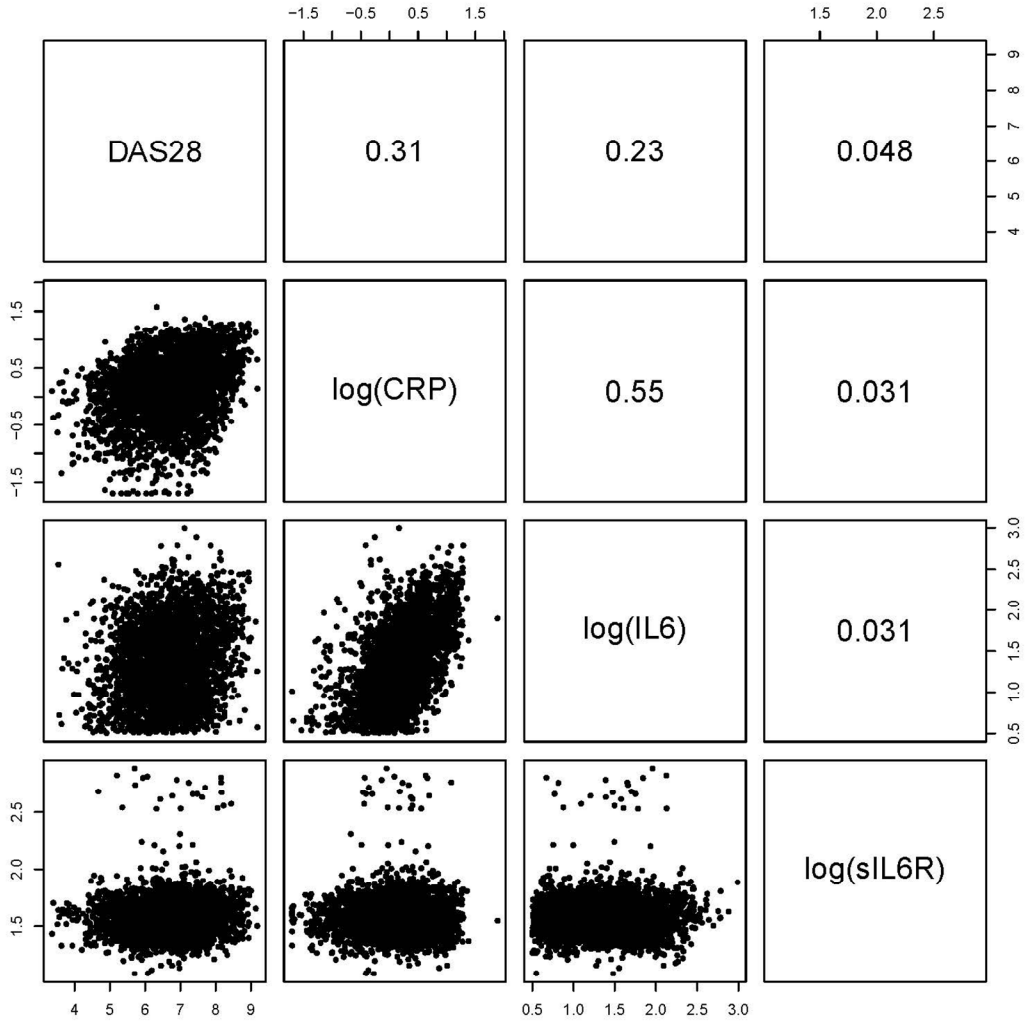
β=regression coefficient, representing the difference in DAS28 corresponding to each copy of minor allele; DAS28=Disease Activity Score using 28 joints; DMARD=disease-modifying anti-rheumatic drug; IL-6R=interleukin-6 receptor; IL-6ST=IL-6 signal transducer; IR=inadequate responder; sIL-6R=soluble IL-6R; TCZ=tocilizumab.
Baseline DAS28 and TCZ dose were included in the model as covariates. Among the seven-probe set in IL-6ST, no association was found. All estimated β values were <0.3

Supplementary Figure 1. Scatter plots showing correlations between the variables.
(A) Pair-wise scatter plot of the three serum IL-6 assays on baseline samples in the TOWARD study. Spearman rank correlation coefficient and sample size are shown in the upper panel.



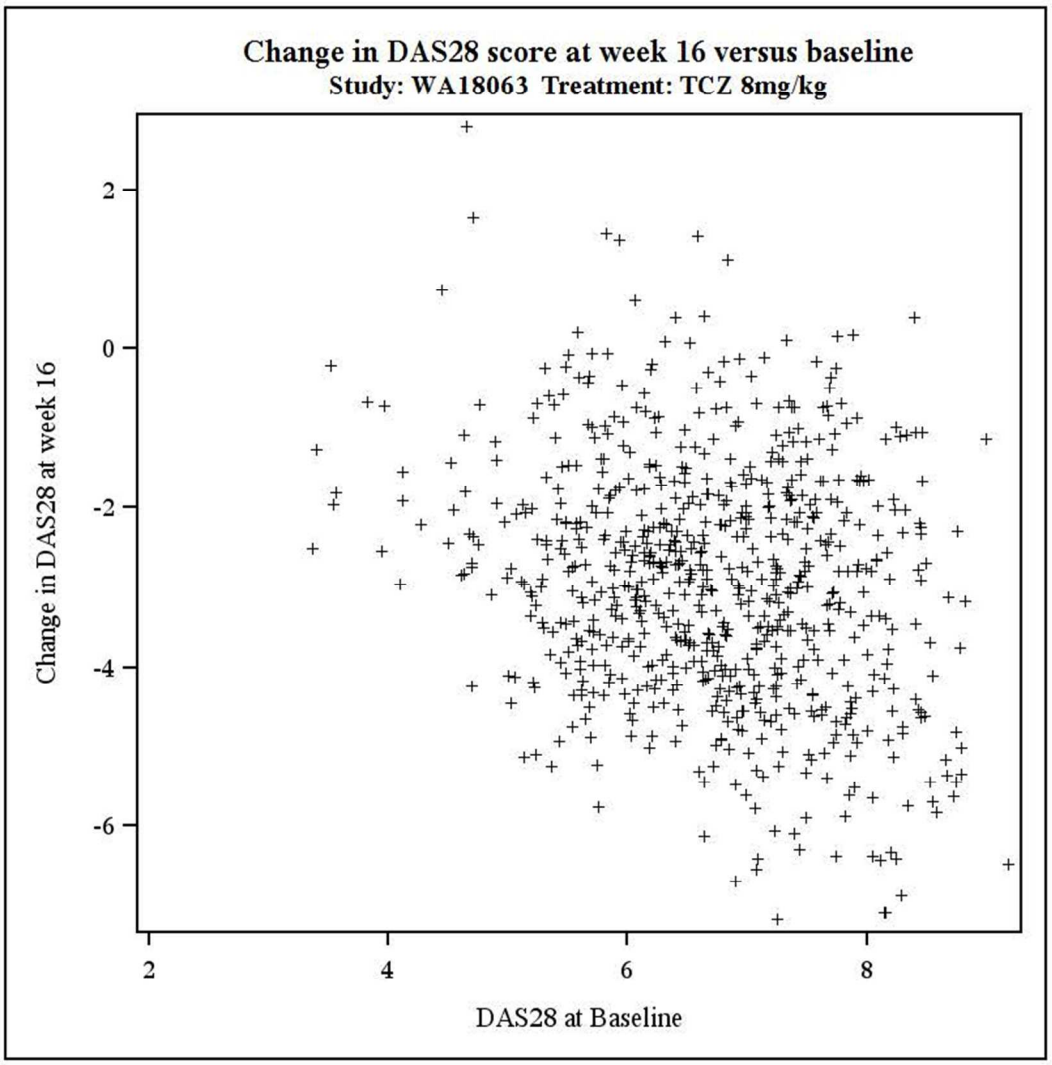
IL-6=interleukin-6; RMB=Rules-Based Medicine assay.

(B) Pair-wise scatter plots between DAS28, CRP, serum IL-6, and serum sIL-6R. Pearson correlation coefficients are labelled in the upper panel.



CRP=C-reactive protein; DAS28=Disease Activity Score at 28 joints; IL-6=interleukin-6; IL-6R=interleukin-6 receptor; sIL-6R=soluble interleukin-6 receptor.

(C) Scatter plot of change in DAS at week 16 versus baseline in tocilizumab-treated patients in the TOWARD study.



DAS=Disease Activity Score; DAS28=Disease Activity Score at 28 joints.

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IL-6 pathway–driven investigation of response to IL-6 receptor inhibition in rheumatoid arthritis

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IL-6 pathway–driven investigation of response to IL-6 receptor inhibition in rheumatoid arthritis

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Clinical Trials Information

Registration names and numbers of tocilizumab clinical trials on Clinicaltrials.gov are OPTION NCT00106548, TOWARD NCT00106574, RADIATE NCT00106522, AMBITION NCT00109408, LITHE NCT00106535 and MEASURE NCT00535782.

ABSTRACT (261 words; maximum, 300)

Objectives To determine whether heterogeneity in interleukin-6 (IL-6), IL-6 receptor and other components of the IL-6 signalling pathway/network, at the gene, transcript and protein levels, correlates with disease activity in patients with rheumatoid arthritis (RA) and with clinical response to tocilizumab.

Design Biomarker samples and clinical data for five phase 3 trials of tocilizumab were analysed using serum (3,751 samples), genotype (927 samples) and transcript (217 samples) analyses. Linear regression was then used to assess the association between these markers and either baseline disease activity or treatment response.

Results Higher baseline serum IL-6 levels were significantly associated (P<0.0001) with higher baseline DAS28, ESR, CRP and HAQ in patients who had inadequate responses to disease-modifying anti-rheumatic drugs (DMARD-IR), patients who had inadequate responses to anti-tumour necrosis factor (aTNF-IR) and patients who were naive/responders to MTX. Higher baseline serum IL-6 levels were also significantly associated with better clinical response to tocilizumab (versus placebo) measured by cDAS28 in the pooled DMARD-IR (P<0.0001) and MTX-naive populations (P=0.04). However, the association with treatment response was weak. A threefold difference in baseline IL-6 level corresponded to only a 0.17-unit difference in DAS28 at week 16. IL-6 pathway SNPs and RNA levels also were not strongly associated with treatment response.

Conclusions Our analyses illustrate that the biological activity of a disease-associated molecular pathway may impact the benefit of a therapy targeting that pathway. However, the variation in pathway activity, as measured in blood, may not be a strong predictor.

These data suggest that the major contribution to variability in clinical responsiveness to therapeutics in RA remains unknown.

Article Summary

Article focus

- This study aimed to determine whether genetic polymorphism and heterogeneity in expression of IL-6 pathway/network components could explain the variability in baseline disease activity and clinical response to tocilizumab in patients with RA

Key messages

- Higher baseline serum IL-6 levels were significantly associated with higher baseline disease activity (ESR, CRP, DAS28 and HAQ scores) in large populations of methotrexate-naive, DMARD-IR and aTNF-IR patients with RA. Higher baseline serum IL-6 levels also showed significant correlation with better clinical response to tocilizumab (cDAS28) in DMARD-IR and MTX-naive RA patients; however, the effect size was small, limiting the clinical usefulness of the marker in predicting treatment benefit. A threefold higher baseline IL-6 concentration corresponded to only an additional 0.17-unit decrease in DAS28 score in the DMARD-IR patients treated with tocilizumab
- None of the genetic polymorphisms in IL-6 or IL-6R showed any association with treatment response to tocilizumab
- No strong predictor of tocilizumab treatment response was identified; thus, the major factors underlying the patient-to-patient variability in clinical

responsiveness to RA therapies remains unknown. This study illustrated well the difficulty of identifying clinically useful predictive biomarkers in a complex chronic disease

Strengths and limitations of this study

- This study included well-characterised RA patients from five large phase 3 controlled clinical trials. Biomarker data were available for more than 3,700 patients. The extensive collection of RNA, DNA and serum samples allowed thorough investigation of the target-related heterogeneity
- This is the first demonstration of a significant association between baseline serum IL-6 levels and baseline disease activity in a large population of patients with RA, extending earlier findings of elevated serum IL-6 levels in RA patients compared with healthy controls
- The current biomarker analysis is focused on clinical usefulness and included only blood samples. Any potentially useful information about local regulation of IL-6 pathway gene transcription and protein level in tissues involved in RA could not be obtained

INTRODUCTION

Interleukin-6 (IL-6) is a pleiotropic cytokine important in autoimmune diseases, including rheumatoid arthritis (RA).[1] High concentrations of IL-6 are found in the synovial fluid and sera of patients with RA. IL-6 involvement in RA pathogenesis includes involvement in B-cell proliferation and cytokine production, matrix metalloproteinase expression, acute-phase response and anaemia.[2]

IL-6 signal transduction occurs through either a classical (*cis*-) or a *trans*-signalling pathway. In *cis*-signalling, IL-6 binds to membrane-bound IL-6 receptor alpha (mIL-6Rα) before forming a trimer with gp130 (encoded by IL-6 signal transducer [*IL-6ST*]). A signalling complex is formed when this heterotrimer forms a dimer with another IL-6/mIL-6R/gp130 complex.[1] However, though gp130 is ubiquitously expressed, mIL-6R expression is restricted to hepatocytes and a subset of leukocytes. The *trans*-signalling pathway makes use of a soluble form of IL-6R (sIL-6R), to which IL-6 binds before forming a heterodimer with membrane-bound gp130, allowing cells that do not express mIL-6R to be activated by IL-6.[1]

Studies of tocilizumab,[3-8] a humanised monoclonal antibody targeted to IL-6R (membrane and soluble) that inhibits the interaction of IL-6 with its receptor, have shown meaningful amelioration of RA. However, the response is heterogeneous, consistent with the responses of other RA therapies. In a pooled analysis of patients who were inadequate responders to disease-modifying anti-rheumatic drug (DMARD-IR) in pivotal trials, 39% of those receiving 8 mg/kg tocilizumab for 24 weeks failed to achieve American College

of Rheumatology (ACR) 20 response (a 20% improvement), whereas 18% did achieve ACR70 response (a 70% improvement). Therefore, there is great interest in discovering biomarkers to aid in physician decision-making by the accurate prediction of clinical response in individual patients.

IL-6 and IL-6R levels vary in the sera of RA patients.[9,10] It has been suggested that some of this heterogeneity is genetically determined. The rs8192284 A/C polymorphism is found at the cleavage site of *mIL-6R* (Gln 357/Asp358) and has been associated with increased sIL-6R levels and RA susceptibility,[11,12] whereas a polymorphism at –174 in the promoter region of *IL-6* (rs1800795) affects transcription activity and, thus, serum levels of IL-6.[13] Beyond RA, IL-6R polymorphisms that reduce cardiovascular risk[14,15] attenuate classical IL-6 signalling by increasing the cleavage rate of mIL-6R (rs2228145 and rs8192284) and mirror the effects of tocilizumab on levels of IL-6 (increase), C-reactive protein (CRP) and fibrinogen (decrease). In asthma, the rs4129267 polymorphism in IL-6R was found to be associated with increased risk, and Ferreira et al[16] suggested that an IL-6R antagonist may show efficacy in asthma in a genotype-dependent manner. The aim of this study was to determine whether baseline heterogeneity in IL-6, IL-6R and other components of the IL-6 signalling pathway/network, at the gene, transcript and protein levels, correlates with disease activity in RA and clinical response to tocilizumab.

METHODS

Patients and samples

Serum specimens used in this study were collected from five pivotal phase 3 studies of tocilizumab in patients with RA: RADIATE,[4] OPTION,[3] TOWARD,[5] AMBITION[6] and LITHE[7] (Table 1). At baseline, serum samples were taken pre-dose and at fasting per protocol, resulting in the vast majority of them collected in the morning. The distribution of the sampling times is shown in Supplementary Figure 2. DNA samples were from RADIATE, OPTION and TOWARD, and RNA samples were from RADIATE. These trials evaluated tocilizumab (4 mg/kg or 8 mg/kg), administered every 4 weeks over a period of 24 weeks or longer, compared with placebo. The study populations differed according to background therapy with methotrexate (MTX) (OPTION, LITHE, RADIATE), any DMARD (TOWARD) or no DMARD (AMBITION). They also differed according to previous inadequate response to anti-tumour necrosis factor (aTNF) agents (RADIATE), MTX (OPTION, TOWARD, LITHE) or neither (AMBITION). In all studies, oral corticosteroids (≤10 mg/day prednisone or equivalent) were permitted if the dose was stable for at least 6 weeks before baseline. The study protocols were approved by relevant institutional review boards or ethics committees, and written informed consent was obtained from each patient. The studies were carried out in full concordance with the principles of the Declaration of Helsinki and with the laws and regulations of the country in which the research was conducted. DNA and RNA samples were collected only from patients who gave separate informed consent. DNA and RNA extraction methods are described in the Supplementary Material.

Serum analysis for IL-6 and sIL-6R

Three assays for serum IL-6 levels were used to analyse samples from TOWARD, as follows: Quantikine Human IL-6 Immunoassay (R&D Systems Inc., Minneapolis, MN), performed by Huntington Life Sciences (Huntington, UK) according to the instructions of the manufacturer (Roche Diagnostics, Penzberg, Germany) of the IL-6 assay[17]; IMPACT platform,[18] performed by Roche Diagnostics; and RBM IL-6 assay, performed by Myriad RBM (Austin, TX). Given the tight correlations among these three assays (Supplementary Figure 1A), we chose to analyse data from the Quantikine Human IL-6 Immunoassay (R&D Systems Inc.) because they were available for nearly all patients in all five core studies. The limit of detection of this IL-6 assay is 3.12 pg/ml. The assay is unaffected by the addition of recombinant human sIL-6R[19]; therefore, it can be assumed that the data reflect the total concentration of IL-6 (i.e. the concentration of free IL-6 plus the amount of IL-6 bound to sIL-6R).

Serum concentrations of endogenous sIL-6R were measured by Huntington Life Sciences using the Quantikine Human sIL-6R Immunoassay (R&D Systems Inc.) according to the manufacturer's instructions. The sensitivity of the sIL-6R assay is 31.2 pg/ml. Measured sIL-6R concentrations are insensitive to the addition of recombinant human IL-6 but are sensitive to the presence of tocilizumab.[20] Therefore, it can be assumed that the sIL-6R data presented reflect the total amount of free sIL-6R plus sIL-6R complexed with IL-6 plus (in samples that contain tocilizumab) an unquantified fraction of sIL-6R bound by tocilizumab.

Genotyping

DNA samples were genotyped using HumanHap550k BeadChip version 3.0 (Illumina, San Diego, CA) and custom chips using the OPA genotyping assay. Quality control (QC) methods for DNA samples and genotyping data are described in the Supplementary Material. High-quality markers with call rates ≥95% were included in the analysis. To reduce the multiplicity of hypothesis testing, a targeted approach was taken. Three groups of genes were considered in this analysis. Tier 1 markers consisted of 26 polymorphisms within the *IL-6* and *IL-6R* regions (Supplementary Table 1). Ten SNPs were selected in the IL-6 region, which is defined as 5 kb upstream and 5 kb downstream of the transcription coordinate; the SNPs cover a region of 12.6 kb. Similarly, 18 SNPs were selected in the IL-6R region, covering a region of 62.2 kb. The association of these markers with treatment benefit represents primary hypotheses. Tier 2 markers consisted of 233 polymorphisms within 22 genes identified as in the IL-6 pathway, termed the IL-6 canonical pathway (Supplementary Material). Tier 3 markers consisted of 1,005 polymorphisms in 67 genes, identified with GeneGo (Carlsbad, CA) software, related to IL-6 signalling, herein termed the IL-6 network (Supplementary Material). Markers for tiers 2 and 3 correspond to the decreasing strength of the hypothesis and the increasing dimensions compared with tier 1 markers. This approach provides greater power in the confirmatory analysis for the primary biomarker hypothesis yet allows for focused exploratory analysis to address the secondary hypotheses.

Polymerase chain reaction (PCR)–based HLA-DRB1 typing was performed on the DNA samples independently of the genotyping using whole genome and custom arrays. The

number of shared epitope alleles (0, 1 or 2) was determined for each patient based on his or her HLA-DRB1 genotype.

Transcript analysis

Two hundred thirty-three RNA samples, prepared from whole blood, at baseline were analysed using the GeneChip® Human Genome U133 Plus 2.0 Array (Affymetrix, Santa Clara, CA). After samples that failed quality control tests were removed (Supplementary Material), data for the remaining 217 samples were normalised using the Robust Multi-Array Analysis normalisation[21] (Bioconductor). Transcripts for *IL-6*, *IL-6R* and *IL-6ST* were extracted from the normalised data. IL-6 transcript levels were not analysed because of the low levels of signal observed. Three probe sets targeting *IL-6R* were analysed. Of those, 205945_at and 217489_at expression levels were found to be strongly correlated (Pearson's r^2=0.75), and the third probe set, 226333_at, had a weaker correlation with the other two (Pearson's r^2=0.34 and r^2=0.38). These three probe sets represent the primary hypothesis in RNA transcripts. In addition, seven probe sets from *IL-6ST* were analysed as secondary markers.

Measures of disease activity and clinical response

Six different measures were used for disease activity at baseline: erythrocyte sedimentation rate (ESR), CRP, swollen joint count at 28 joints (SJC28), tender joint count at 28 joints (TJC28) and Health Assessment Questionnaire (HAQ), with disease activity at 28 joints (DAS28; derived from the four core components of ESR, SJC, TJC and patient global assessment) considered the primary end point. As a measurement of

treatment response, change in DAS28 from baseline at week 16 (cDAS28) was mainly used. Unlike dichotomous responder criteria (e.g. ACR response, DAS28 remission and clinical disease activity index remission), this continuous measure captures the range of individual responses and is therefore more sensitive in detecting the effect of biomarkers across different levels of prevalence. In all studies except AMBITION, patients were allowed to enter escape therapy at 16 weeks if they had inadequate responses. Therefore, cDAS28 was used at week 16 to increase the sample size and to minimise bias.

Statistical analysis

Table 2 provides a summary of all markers analysed. Quality control analyses were performed for genotyping and gene expression data, as described in the Supplementary Material; assays and samples with poor data quality were removed. Distribution was assessed for each marker, and appropriate transformation was applied before further analysis. Minor allele frequencies and Hardy-Weinberg equilibrium were calculated for single nucleotide polymorphisms (SNPs). Given that the distribution within each gene expression probe set was approximately normal, no further transformation was applied. Log transformation was applied to the protein markers IL-6 and sIL-6R because of the skewed distribution of raw values.

Correlations among the baseline markers were assessed within each sample type to enable understanding of the amount of information shared among the assays. For SNPs, linkage disequilibrium coefficients within IL-6 and IL-6R were obtained from HapMap 22 based on the CEU (Utah residents of Northern and Western European ancestry from

the CEPH collection) data. For the gene expression markers, correlation coefficients were calculated among probe sets within *IL-6R* and *IL-6ST*. For the proteins, correlation was calculated among the three assays for IL-6 and between IL-6 and sIL-6R. Pearson or Spearman rank correlation was used as appropriate.

Linear regression was used to assess the association between markers with baseline disease activity and markers with treatment response. Because of the skewed distribution of the raw values, log(ESR) and log(CRP+1) were used in the regression analysis. All patients were included in the regression analysis of baseline disease activity. In the regression analysis of cDAS28, separate analyses were undertaken in patients treated with tocilizumab and those administered placebo. Given that baseline DAS28 is strongly associated with change in DAS28 at week 16, it was included as a covariate in all models. No additional covariates were included in the model. In addition, the number of shared epitope alleles[22] (0, 1 or 2) and the tocilizumab dose were used as covariates in the analysis of genetic data.

The numbers of genetic markers tested were 18, 233 and 1,004, respectively, for the tier 1, 2 and 3 markers. In addition, six baseline end points were used on the baseline disease association analysis. Analyses were undertaken in the white population and the all-patient population. To adjust for multiple testing, the false discovery rate (FDR)[23] was calculated within each tier and within the white population and the all-patient population. This FDR accounted for the multiplicity of markers and the end points.

To help interpret the outcome of the regression analysis and to enable direct comparison of the analysis results across all markers, consistent representations of the results were used. For continuous markers, the regression coefficients were standardised to reflect how many units of change in the end points corresponded to a difference of 1 standard deviation (SD) in the marker value. For the SNP markers, an allelic model was used (i.e. two copies of the minor allele had twice the effect of one copy). The regression coefficient corresponded to the change in end point per copy of minor allele.

RESULTS

Baseline characteristics of patients with DNA, RNA and serum samples

Baseline characteristics of the patient subgroups with DNA, RNA and serum samples available are shown in Table 1. They are generally comparable to the overall population for each study. Patient ethnicity is relevant to the genetic analysis. Therefore, the proportion of patients of European ancestry (White) is shown for the DNA subpopulations. Of the 927 patients with DNA samples, 730 (79%) were of European ancestry (White) (Table 1). As expected based on treatment experience, patients from the MTX-naive or the MTX-free study had shorter disease duration, and those from the aTNF-IR study had longer disease duration. For serum markers where large sample size was available, data were analysed separately for the MTX-naive/MTX-free population, pooled DMARD-IR population and aTNF-IR population. The distribution of baseline IL-6 was similar in the rheumatoid factor–positive and –negative subpopulations (45.2 ± 60.5 and 41.5 ± 60.6 [mean ± SD], respectively).

Associations with baseline disease activity

Higher baseline serum IL-6 levels were significantly associated with higher baseline DAS28, ESR, CRP and HAQ in all three populations analysed, with P<0.0001 in most cases (Table 3). The strongest association was observed between CRP and IL-6, for a correlation coefficient of 0.29 to 0.36 in three populations (Supplementary Figure 1B). IL-6 was not strongly associated with SJC28 or TJC28. Baseline sIL-6R was not strongly associated with any baseline disease activity measures.

Among the baseline RNA transcripts, an association was observed between the IL-6R transcript and CRP and ESR at baseline (data not shown). No association was found with the other baseline disease activity measures. Association with CRP and ESR was also observed, to a lesser degree, for an IL-6ST transcript.

After correction for multiple testing, no significant associations were found for any of the *IL-6* or *IL-6R* polymorphisms genotyped with baseline disease activity. Of the 1,237 polymorphisms representing 22 genes in the IL-6 canonical pathway, 67 additional genes in the IL-6 network, six baseline disease activity end points and two patient populations (all-patient, white), six associations were found to have borderline significance after adjusting for multiple testing, with an FDR of approximately 5%. There were SNPs in *TOLLIP* and *RUNX1* versus ESR and SNPs in *IL1RAPL1* versus SJC and ESR. Such borderline significance meant the association should be treated as a weak hypothesis.

Associations with clinical response to tocilizumab

Higher baseline serum IL-6 concentrations were significantly associated with better clinical response to tocilizumab but not to placebo, as measured by cDAS28 in the pooled DMARD-IR population (P<0.0001) (Supplementary Table 2, Figure 1). The same effect was observed in the MTX-naive population (P=0.04). In the aTNF-IR population, the association was not significant, partially because of the smaller sample size. In the DMARD-IR population, a threefold difference in baseline IL-6 concentration, equivalent to 1 SD in the baseline log(IL-6) distribution, corresponded to a small difference of 0.17 units in cDAS28 score at week 16. The association between serum IL-6 level and achievement of DAS28 remission (DAS28 <2.6) was analysed using receiver operating characteristic analysis in the pooled DMARD-IR population treated with tocilizumab (n=1,547). The area under the curve was only 0.59, suggesting that baseline serum IL-6 levels provided very little discrimination between those achieving and those not achieving DAS28 remission. In the placebo group, the direction of association was opposite that of tocilizumab-treated patients, indicating that baseline serum IL-6 level (with no longitudinal data on change in level) is not a prognostic factor for clinical response in RA in these cohorts.

Baseline sIL-6R concentration in serum had no effect on treatment response in either the tocilizumab or the placebo group (Supplementary Table 2). IL-6R whole blood mRNA (in RADIATE) was not associated with clinical response to tocilizumab or placebo treatment (Supplementary Table 2). No *IL-6* or *IL-6R* polymorphisms were associated with cDAS28 after correction for multiple testing. Although rs2069840 and rs12700386

had a raw P<0.05 (Table 4), the estimated effect size was small. Each copy of the minor allele for rs2069840 corresponded to only a 0.3-unit difference in cDAS28. Of the SNPs in the canonical and the network or signalling pathway, rs973767 (an intronic SNP in *PTPN2*) had the lowest P value (5.7×10^{-5}) but was not significant after correction for multiple testing (FDR=0.057; Table 4). In the rheumatoid factor–positive subpopulation, the effect of baseline serum IL-6 on clinical response was very similar to that in the overall population (Supplementary Figure 3).

DISCUSSION

We have investigated the associations among a number of DNA, RNA and protein biomarkers directly related to IL-6 signalling (Table 2) with baseline disease activity and treatment benefit from tocilizumab (Table 1). We demonstrate the modest association between serum IL-6 levels and clinical disease activity as measured by DAS28 and HAQ. Although serum IL-6 levels have previously been shown to be elevated in patients with RA compared with controls,[9,24] this is the first time a significant correlation between IL-6 level and disease activity has been demonstrated in a large population. IL-6 level accounted for only a small component of observed variance in disease activity (Supplementary Table 2, Figure 1). Of the four DAS28 components, serum IL-6 level was most strongly associated with ESR/CRP. This is not surprising given the pivotal role of IL-6 (and the pronounced inhibitory effect of tocilizumab) on the acute-phase response.[25] Interestingly, HAQ, a measure of patient-reported disability independent of inflammation, also had a significant association with serum IL-6 level.

Littman[26] proposes that IL-6/tocilizumab may represent 'low-hanging fruit' in terms of personalised health care in RA, but our analysis suggested otherwise. Higher baseline DAS28 was associated with higher change in DAS28 in response to therapy (Supplementary Figure 1C). When change in DAS28 was corrected for baseline DAS28, only a weak association with serum IL-6 was observed. Even though IL-6 has been shown to decrease with effective treatments, such as modified-release prednisone,[27] in RA, we saw no evidence that a single baseline 'snapshot' serum IL-6 level was a general prognostic factor for clinical response in RA (i.e. predicted subsequent treatment response or disease course).

Serum IL-6 exhibits a pronounced circadian rhythm.[28] This source of variability was minimised, but not eliminated, by collecting the samples in as narrow a time window as achievable in large clinical studies. It is possible that discordance in IL-6 signalling activity between blood and synovial tissue explains the lack of association with blood samples in our study. However, this cannot explain the negative genetic data. The lack of genetic associations between polymorphisms in *IL-6R* (including those determining sIL-6R levels) and baseline disease activity[11,29] shows that sIL-6R levels, though highly variable, are not appreciably different between patients who have RA and those who are healthy.[30,31] The effect of a genetic marker can be affected by a patient's genetic background. An ethnically homogeneous population is ideal for genetic analysis. In this cohort, 79% of the patients were of European ancestry (White). All genetic analyses were performed in the overall population for maximum sample size and in the White

subpopulation for genetic homogeneity. The White subpopulation was sufficiently large; therefore, the lack of a strong genetic effect is compelling.

Formal multiplicity adjustment was not carried out in the analysis of serum and RNA markers. However, informal assessment was used to help interpret the results of the statistical analysis. Multiplicity of the statistical testing was indicated by multiple markers and multiple end points; the numbers of markers in each sample type are shown in Table 2. Six baseline disease characteristics and one clinical response end point were used. In addition, genetic analyses were performed in the White and the overall populations. Overall, ≥10 tests were carried out for each serum and RNA marker; results with $P<0.0001$ would have been significant had adjustment for multiplicity been performed, and results with unadjusted $P>0.01$ can be regarded as likely false positives.

Power assessment was not performed. Empirically, given the large sample size for DNA and serum samples, the power to detect a clinically relevant effect at $\alpha=0.05$ was very high. Therefore, $P>0.05$ can be interpreted as evidence of no effect. Analyses of RNA markers had low power because of the limited sample size.

Our analyses illustrate that in a disease such as RA, variation in the abundance of a therapeutic target may not be a strong predictor of patient benefit. In particular, the response to tocilizumab was not dependent on IL-6R SNPs, which were found to be associated with asthma and cardiovascular risk. In addition, a genome-wide association approach using 1,600 patients from five controlled clinical trials did not yield any strong

predictor to tocilizumab treatment response.[32] These data suggest that the major contribution to variability in clinical responsiveness to therapeutics in RA remains unknown, and they illustrate the challenges of identifying predictive biomarkers, even in large, well-conducted studies.

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Competing interests

All authors are (or were at the time of the study) employees of Roche or Genentech, a member of the Roche group, and four authors (AH, AK, OH and RU) also own stock in Roche.

Author contributions

JW designed the study, conducted research, conducted the literature search, analysed and interpreted the data and drafted the manuscript; AP designed the study, conducted research, conducted the literature search, collected data, analysed and interpreted the data, oversaw data collection and drafted the manuscript; RU analysed and interpreted the data and drafted the manuscript; SG designed the study and collected data; GL analysed and interpreted the data; CR analysed and interpreted the data; RB designed the study, collected data and analysed the data; AK analysed and interpreted the data; AH designed the study and collected data; MM designed the study; OH interpreted the data and drafted the manuscript.

All authors revised the manuscript critically for important intellectual content and approved the final version for submission.

Ethics approval

The study protocols were approved by relevant institutional review boards or ethics committees, and written informed consent was obtained from each patient. The studies were carried out in full concordance with the principles of the Declaration of Helsinki and with the laws and regulations of the country in which the research was conducted. DNA and RNA samples were collected only from patients who gave separate informed consent.

Data sharing

No additional data are available at this time.

FIGURE

Figure 1. Serum IL-6 concentration association with response to treatment. Forest plots show the effect and 95% CI for the association of IL-6 with cDAS28 at 16 weeks across treatment lines. cDAS28, change in DAS28 from baseline at week 16; CI, confidence interval; DMARD, disease-modifying antirheumatic drug; IR, inadequate responder; MTX, methotrexate; SD, standard deviation; TCZ, tocilizumab; TNF, tumour necrosis factor. •, MTX/DMARD; •, TCZ+MTX/DMARD.

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TABLES

Table 1. Demographics of patients from whom biological samples were collected and analysed and whose data passed a quality control check

Sample type	DNA			RNA	Serum				
Study	**OPTION**	**RADIATE**	**TOWARD**	**RADIATE**	**OPTION**	**LITHE**	**TOWARD**	**AMBITION**	**RADIATE**
Patient population	MTX-IR	aTNF-IR	DMARD-IR	aTNF-IR	MTX-IR	MTX-IR	DMARD-IR	MTX naive or free	aTNF-IR
Treatment	8 mg/kg TCZ + MTX	8 mg/kg TCZ + MTX	8 mg/kg TCZ + DMARD	8 mg/kg TCZ + MTX	8 mg/kg TCZ + MTX	8 mg/kg TCZ + MTX	8 mg/kg TCZ + DMARD	8 mg/kg TCZ + MTX	8 mg/kg TCZ + MTX
	4 mg/kg TCZ + MTX	4 mg/kg TCZ + MTX		4 mg/kg TCZ + MTX	4 mg/kg TCZ + MTX	4 mg/kg TCZ + MTX			4 mg/kg TCZ + MTX
	MTX	MTX	DMARD	MTX	MTX	MTX	DMARD	MTX	MTX
Total patients, n	286	178	463	217	603	1126	1010	581	431
TCZ, n	189	119	301	151	409	753	661	244	289
MTX, n	97	59	162	66	194	373	349	337	142
Age, years	51.3 (11.1)	51.5 (12.4)	54.2 (12.6)	53.2 (11.9)	51.0 (12.2)	51.9 (12.4)	53.4 (12.9)	50.3 (13.0)	52.9 (12.6)
Female, %	79.0	79.8	81.0	82.40	81.8	82.9	81.7	80.4	81.4
Height, cm	163 (9)	165 (8)	164 (9)	165 (8)	162 (9)	162 (9)	163 (9)	163 (9)	165 (8)
Weight, kg	73 (17)	77 (19)	76 (18)	77 (20)	79 (17)	73 (19)	74 (19)	73 (18)	75 (18)
RA duration, years	7.8 (7.3)	11.1 (8.3)	9.7 (9.1)	12.1 (9.6)	7.6 (7.3)	9.2 (8.0)	9.6 (8.8)	6.4 (8.2)	11.6 (9.1)
RF positive, %	76.2	79.8	75.6	76.5	77.6	82.1	77.1	72.5	75.2
White, %	73.8	93.3	76.2	Not applicable			Not applicable		
TCZ, n	141	109	233						
MTX, n	70	57	120						
DAS28	6.8 (0.9)	6.8 (1)	6.6 (1)	6.8 (0.9)	6.8 (0.9)	6.5 (0.9)	6.7 (1.0)	6.8 (0.9)	6.8 (1.0)
HAQ	1.6 (0.6)	1.7 (0.6)	1.5 (0.6)	1.7 (0.6)	1.6 (0.6)	1.5 (0.6)	1.5 (0.6)	1.5 (0.6)	1.7 (0.6)
SJC	20.9 (11.3)	18.8 (10.6)	20.5 (11.9)	18.1 (9.8)	20.0 (11.2)	16.9 (9.4)	19.6 (11.5)	19.8 (11.2)	18.9 (10.6)
TJC	32.3 (15.2)	31.2 (15.2)	30.1 (15.8)	31.4 (15.1)	32.6 (15.6)	28.3 (14.7)	30.0 (15.7)	31.9 (14.4)	31.1 (15.6)
CRP	2.4 (2.9)	3.7 (4.2)	2.3 (2.9)	3.3 (3.8)	2.6 (3.0)	2.2 (2.5)	2.6 (3.8)	3.0 (3.3)	3.2 (3.8)

aTNF, anti-tumour necrosis factor; CRP, C-reactive protein; DAS28 , Disease Activity Score using 28 joints; DMARD, disease-modifying antirheumatic drug; HAQ, Health Assessment Questionnaire; IR, inadequate responder; MTX, methotrexate; RA, rheumatoid arthritis; RF, rheumatoid factor; SJC, swollen joint count; TCZ, tocilizumab; TJC, tender joint count.

Values with numbers in parentheses are mean (SD).

Table 2. Summary of markers investigated. For the DNA markers, SNPs in IL-6 and IL-6R are considered tier 1, IL-6 network genes are considered tier 2, and IL-6 pathway genes are considered tier 3

Sample type	Target gene	Analytes, n	Assay (manufacturer)
Primary			
DNA	*IL-6*	10 SNPs	Bead-Chip arrays HumanHap550k version 3.0, OPA custom array (Illumina)
	IL-6R	18 SNPs	
RNA	*IL-6R*	3 probe sets	GeneChip Human Genome U133 Plus 2.0 Array (Affymetrix)
Serum	*IL-6*	1	Human IL-6 Immunoassay (Quantikine)
	sIL-6R	1	Human sIL-6R Immunoassay (Quantikine)
Secondary			
DNA	IL-6 network genes	233 SNPs (22 genes)	Bead-Chip arrays HumanHap550k version 3.0, OPA custom array (Illumina)
	IL-6 pathway genes	1004 SNPs (67 genes)	
RNA	*IL-6ST*	7 probe sets	GeneChip Human Genome U133 Plus 2.0 Array (Affymetrix)

IL-6, interleukin 6; IL-6R, IL-6 receptor; IL-6ST, IL-6 signal transducer; sIL-6R, soluble IL-6R; SNP, single-nucleotide polymorphism.

Table 3. Association of markers with baseline disease activity. All results for serum markers are shown. For RNA, the probe sets within each gene were strongly correlated. One probe set each in IL-6R and IL-6ST is shown. For DNA, only associations with raw P<0.01 (tier 1) and FDR P<0.05 (tiers 2 and 3) were shown. FDR was calculated within the population (white or all) and within the tier

Serum protein and RNA markers									
		Serum IL-6			Serum sIL-6R			IL-6R 217489_s_at	IL-6ST 234967_at
End point		AMBITION	RADIATE	Pooled DMARD-IR	AMBITION	RADIATE	Pooled DMARD-IR	RADIATE	RADIATE
DAS28	n	480	322	2286	577	423	2620	217	217
	β	0.209	0.257	0.201	–0.007	0.075	0.054	0.109	–0.04
	r^2	0.051	0.072	0.044	0	0.006	0.003	0.013	0.002
	p	<0.0001	<0.0001	<0.0001	0.8478	0.1018	0.0044	0.09	0.54
TJC	n	481	327	2305	580	428	2642	217	217
	β	–0.186	1.455	0.138	–0.154	1.28	0.054	0.27	1.276
	r^2	0	0.009	0	0	0.007	0	0	0.007
	p	0.7742	0.0938	0.6611	0.7967	0.0889	0.8558	0.79	0.2140
SJC	n	481	327	2305	580	428	2642	217	217
	β	0.247	1.421	0.813	–0.679	–-0.069	0.52	–0.864	0.218
	r^2	0	0.018	0.006	0.004	0	0.002	0.008	0
	p	0.6279	0.0158	0.0003	0.1424	0.8938	0.013	0.1950	0.7440
ESR	n	481	327	2299	580	428	2635	217	217
	β	0.19	0.172	0.151	0.039	–0.003	0.023	0.164	–0.132
	r^2	0.088	0.091	0.055	0.004	0	0.001	0.082	0.053

	p	<0.0001	<0.0001	<0.0001	0.132	0.9158	0.0672	<0.0001	0.0007
CRP	n	481	327	2305	580	428	2642	217	217
	β	0.363	0.429	0.35	0.007	0.019	0.019	0.228	–-0.145
	r^2	0.288	0.358	0.309	0	0.001	0.001	0.089	0.036
	p	<0.0001	<0.0001	<0.0001	0.8103	0.5935	0.1242	<0.0001	0.005
HAQ	n	480	324	2165	579	425	2465	217	217
	β	0.085	0.145	0.084	0.002	0.074	0.024	0.048	–0.103
	r^2	0.019	0.062	0.018	0	0.016	0.002	0.007	0.031
	p	0.0022	<0.0001	<0.0001	0.933	0.0089	0.0504	0.22	0.009

DNA markers

Tier	Gene	Chr	SNP	Population	MAF	End point	n	β	SE	Raw P	FDR P
1	*IL-6R*	1	rs1386821	White	0.17	HAQ	708	0.135	0.041	0.001	0.18
				All	0.15	HAQ	845	0.118	0.040	0.004	0.52
3	*TOLLIP*	11	rs5743899	All	0.22	ESR	893	0.15	0.03449	1.5E-05	0.046
	RUNX1	21	rs2252585	All	0.32	ESR	905	0.1209	0.02884	3.0E-05	0.046
	IL-1RAPL1	23	rs12559028	All	0.49	SJC	905	2.182	0.5349	4.9E-05	0.050
			rs5943618	All	0.44	SJC	905	2.27	0.5424	3.1E-05	0.046
			rs4829239	All	0.38	SJC	905	2.321	0.5592	3.6E-05	0.046
			rs5927671	All	0.45	ESR	897	0.1181	0.02853	3.8E-05	0.046

β, regression coefficient, representing the difference in the end point corresponding to standard deviation in marker value; Chr, chromosome; CRP, C-reactive protein; DAS28, Disease Activity Score using 28 joints; DMARD, disease-modifying antirheumatic drug; ESR, erythrocyte sedimentation rate; HAQ, Health Assessment Questionnaire; IL-6ST, IL-6 signal transducer; IR, inadequate responder; MAF, minor allele frequency; MTX, methotrexate; SJC, swollen joint count; TCZ, tocilizumab; TJC, tender joint count.

Table 4. Association of baseline markers with treatment response measured by change in DAS28 from baseline at week 16. Baseline DAS28 and TCZ dose were included in the model as covariates. FDR was calculated within the white or all population and within the tier

All association results for primary DNA markers											
Gene	SNP	All DMARD-IR, TCZ					White DMARD-IR, TCZ				
		MAF	n	β	SE	Raw P	MAF	n	β	SE	Raw P
IL-6	rs12700386	0.19	583	0.22	0.11	0.049	0.18	468	0.16	0.13	0.20
	rs2069833	0.35	584	–0.08	0.08	0.34	0.42	469	–0.13	0.09	0.18
	rs2069837	0.08	586	–0.11	0.15	0.44	0.08	471	–0.05	0.16	0.75
	rs2066992	0.12	586	–0.09	0.12	0.49	0.06	471	–0.11	0.21	0.61
	rs2069840	0.32	584	0.24	0.09	0.007	0.33	469	0.30	0.10	0.0026
	rs1554606	0.4	586	–0.10	0.08	0.23	0.45	471	–0.15	0.09	0.12
	rs2069845	0.4	583	–0.10	0.08	0.23	0.45	468	–0.15	0.09	0.12
	rs2069861	0.07	584	–0.10	0.15	0.53	0.08	469	–0.11	0.16	0.50
	rs10242595	0.36	586	–0.02	0.08	0.86	0.32	471	–0.02	0.10	0.80
	rs11766273	0.07	586	0.10	0.16	0.53	0.08	471	0.05	0.17	0.75
IL-6R	rs1386821	0.15	586	0.02	0.12	0.87	0.17	471	0.06	0.13	0.67
	rs4075015	0.39	584	–0.06	0.09	0.50	0.41	469	–0.07	0.10	0.46
	rs6684439	0.41	586	0.03	0.08	0.76	0.39	471	0.06	0.10	0.55
	rs4845618	0.43	582	0.03	0.08	0.74	0.44	467	0.00	0.09	0.99
	rs8192282	0.15	581	–0.09	0.12	0.46	0.16	468	–0.08	0.13	0.53
	rs7549250	0.43	575	0.07	0.08	0.43	0.44	462	0.02	0.09	0.81
	rs4553185	0.43	586	0.04	0.08	0.60	0.44	471	0.01	0.09	0.90
	rs4845623	0.44	586	–0.01	0.08	0.92	0.41	471	0.01	0.10	0.89

	rs4537545	0.44	586	–0.04	0.08	0.60	0.41	471	–0.01	0.10	0.93
	rs4129267	0.41	586	–0.02	0.08	0.77	0.4	471	0.02	0.10	0.87
	rs8192284	0.41	582	–0.03	0.08	0.76	0.4	467	0.02	0.10	0.87
	rs11265618	0.17	586	–0.05	0.11	0.64	0.17	471	–0.04	0.13	0.73
	rs4329505	0.16	582	–0.03	0.11	0.81	0.16	467	–0.02	0.13	0.87
	rs4240872	0.24	586	0.03	0.10	0.79	0.23	471	–0.01	0.11	0.93
	rs4509570	0.24	580	0.02	0.10	0.85	0.23	466	–0.02	0.11	0.83
	rs2229238	0.19	584	–0.03	0.11	0.75	0.19	469	0.00	0.12	0.98
	rs7514452	0.19	586	–0.03	0.11	0.77	0.19	471	0.00	0.12	0.98
	rs7526293	0.2	584	–0.04	0.10	0.71	0.2	469	–0.03	0.12	0.79

DNA markers analyzed in the pooled DMARD-IR population with an FDR* of <0.1

Tier	Gene	SNP	Population	MAF	n	β	SE	RAW P	FDR P
1	*IL-6*	rs2069840	White	0.33	469	0.30	0.10	0.0026	0.073
3	*PTPN2*	rs973767	All	0.14	586	0.48	0.12	7.2E-05	0.072
			White	0.16	471	0.53	0.13	5.7E-05	0.057

β, regression coefficient, representing the difference in DAS28 corresponding to each SD in marker value; DAS28, Disease Activity Score using 28 joints; DMARD, disease-modifying antirheumatic drug; FDR, false discovery rate; IL-6, interleukin-6; IL-6R, IL-6 receptor; IR, inadequate responder; MAF, minor allele frequency; SNP, single nucleotide polymorphism; TCZ , tocilizumab.

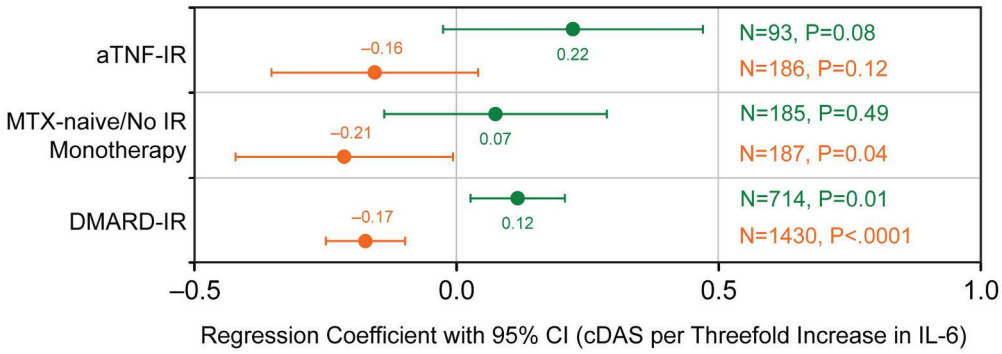


Figure 1. Serum IL-6 concentration association with response to treatment. Forest plots show the effect and 95% CI for the association of IL-6 with cDAS28 at 16 weeks across treatment lines. cDAS28, change in DAS28 from baseline at week 16; CI, confidence interval; DMARD, disease-modifying antirheumatic drug; IR, inadequate responder; MTX, methotrexate; SD, standard deviation; TCZ, tocilizumab; TNF, tumour necrosis factor. •, MTX/DMARD; •, TCZ+MTX/DMARD.
594x228mm (300 x 300 DPI)

SUPPLEMENTARY MATERIAL

Identification of genes on IL-6 canonical pathway and signalling network

IL-6 canonical (i.e. pre-built) pathway genes were identified using IL-6 as a seed query of the canonical GeneGo immune response, and signalling network genes were identified using IL-6 as a seed query of the chemokine/cytokine signalling networks. Relevant genes were selected based on the relative strength of literature support for those interactions.

The 22 canonical pathway genes (excluding IL-6 and IL-6R) were: *ADAM10, ADAM17, CEBPB, CEBPD, IL6ST, JAK1, MAP2K1, MAP2K2, MAPK1, MAPK3, NFKB1, NFKB2, NR3C1, PTPN11, RAF1, REL, RELA, RELB, SOCS3, SOS1, SOS2,* and *STAT3*.

The 67 IL-6 signalling network genes (excluding IL6, IL6R and canonical pathway genes) were: *TNFRSF4, MTHFR, IL10, ATF3, IL1R2, IL1R1, IL1RL2, IL1RL1, IL1F9, IL1RN, STAT1, CTLA4, FN1, IRAK2, MYD88, CD86, IL1RAP, TLR1, RFC1, SPP1, TLR2, PIK3R1, IRF1, IL4, EGR1, TNF, MAPK14, NFKBIE, IRAK1BP1, MAP3K7, IFNGR1, ESR1, RAC1, TRIP6, IRF5, IKBKB, MYC, TLR4, CHUK, TOLLIP, TRAF6, RELT, IL10RA, TIRAP, STAT6, IRAK3, NFKBIA, SOCS1, UBB, NOS2, STAT5A, MAP3K14, TYMS, PTPN2, BCL2, ELAVL1, TYK2, PIK3R2, NFKBIB, BCL2L1, IL10RB, IFNGR2, RUNX1, IL1RAPL1, BTK, IRAK1,* and *IKBKG*.

The polymorphism IDs are available from the corresponding author upon request.

Method for genotyping data QC

Before genotyping, a Y-chromosome test assay was performed for all samples. Sex results of this assay were compared with clinical data, and only samples with concordance in sex assignment were selected for genotyping. DNA samples genotyped using the Illumina standard arrays or custom arrays and with call rates <99% or <95%, respectively, were re-genotyped. Re-assayed samples with call rates consistently below the threshold were removed from the analysis. Sample pairs with kinship coefficient ≥0.5 were

excluded. Markers with call rates <95% were individually reviewed and re-clustered. The quality of markers with call rates ≥95% was assessed using Infinium genotyping QC metrics (Illumina). Single-nucleotide polymorphisms (SNPs) with either >5% missing data or with a minor allele frequency <1% were excluded from the analysis. Chi-square tests of Hardy-Weinberg equilibrium were conducted in white patients; the results were used, along with estimates of minor allele frequency, to assist in the interpretation of associations.

Method for DNA and RNA sample preparation

DNA was extracted from EDTA whole blood using a silica gel-based extraction method (MagNA Pure LC DNA Isolation KIT I, Roche Molecular Systems). DNA was quantified by picogreen fluorescence measurement (Quant-It DNA Assay kit, Invitrogen) and normalized to 50 ng/ul.

Total RNA was prepared from whole blood collected in paxgene tubes using a Qiagen BioRobot MDx. RNA was quantified by ribogreen fluorescence measurement (Quant-It Ribogreen RNA Assay kit, Invitrogen) and normalized to 100 ng/ul.

Method for gene expression data QC

Quality control metrics on MAS5 normalized data, box plots, MvA plots, and principal component analyses were performed using the Bioconductor QC method; 11 chips were identified as having poor quality. Five samples were further excluded because of missing Disease Activity Score using 28 joints (DAS28) erythrocyte sedimentation rate (ESR) data at baseline or week 16.

Supplementary Table 1.SNPs on IL-6 and IL-6R SNPs in the primary analysis and the pair-wise linkage disequilibrium coefficient (R^2) based on data from HapMap22.

Gene: IL6, Chromosome 7			Pair-wise R^2								
SNP ID	**Position**	**Function**	**rs1270038**	**rs2069833**	**rs2069837**	**rs2066992**	**rs2069840**	**rs1554606**	**rs2069845**	**rs2069861**	**rs10242595**
rs12700386	22,729,534	5' upstream									
rs2069833	22,734,189	Intronic	0.134								
rs2069837	22,734,552	Intronic	0.021	0.079							
rs2066992	22,734,774	Intronic	0.013	0.048	0.003						
rs2069840	22,735,097	Intronic	0.333	0.512	0.033	0.02					
rs1554606	22,735,232	Intronic	0.149	0.935	0.084	0.051	0.548				
rs2069845	22,736,674	Intronic	0.149	0.935	0.084	0.051	0.548	1			
rs2069861	22,738,179	3' downstream	0.021	0.065	0.005	0.003	0.033	0.06	0.06		
rs10242595	22,740,756	3' downstream		0.146	0.235	0.143	0.006	0.162	0.162	0.022	
rs11766273	22,742,188	3' downstream		0.11	0.009	0.005	0.056	0.103	0.103	0.009	0.037

Gene IL-6R, Chromosome 1			Pair-wise R^2															
SNP ID	**Position**	**Function**	**rs1386821**	**rs4075015**	**rs6684439**	**rs4845618**	**rs8192282**	**rs7549250**	**rs4553185**	**rs4845623**	**rs4537545**	**rs4129267**	**rs8192284**	**rs1126561**	**rs4329505**	**rs4240872**	**rs4509570**	**rs22923**
rs1386821	152,648,673	Intronic																
rs4075015	152,655,820	Intronic	0.169															
rs6684439	152,662,463	Intronic	0.012	0.04														
rs4845618	152,666,639	Intronic	0.003	0.028	0.033													
rs8192282	152,668,303	Intronic	0.008	0.011	0.013	0.219												
rs7549250	152,670,960	Intronic	0.001	0.013	0.029	0.905	0.198											
rs4553185	152,677,579	Intronic	0.001	0.013	0.029	0.905	0.198	1										
rs4845623	152,682,401	Intronic	0.009	0.048	0.07	0.411	0.085	0.504	0.504									
rs4537545	152,685,503	Intronic	0.003	0.042	0.073	0.393	0.082	0.486	0.486	0.964								

rs4129267	152,692,888	Intronic	0.003	0.042	0.073	0.393	0.082	0.486	0.486	0.964	1							
rs8192284	152,693,594	Intronic	0.002	0.034	0.07	0.368	0.085	0.455	0.455	0.928	0.964	0.964						
rs11265618	152,696,716	Intronic	0.005	0.011	0.012	0.194	0.887	0.176	0.176	0.101	0.098	0.098	0.101					
rs4329505	152,699,044	Intronic	0.005	0.011	0.012	0.194	0.887	0.176	0.176	0.101	0.098	0.098	0.101	1				
rs4240872	152,702,819	Intronic	0.043	0.095	0.009	0.316	0.071	0.356	0.356	0.18	0.205	0.205	0.18	0.087	0.087			
rs4509570	152,703,008	Intronic	0.043	0.095	0.009	0.316	0.071	0.356	0.356	0.18	0.205	0.205	0.18	0.087	0.087	1		
rs2229238	152,704,520	3' UTR	0.026	0.034	0.004	0.254	0.074	0.285	0.285	0.125	0.148	0.148	0.155	0.066	0.066	0.752	0.752	
rs7514452	152,704,708	3' UTR	0.028	0.027	0.005	0.23	0.077	0.26	0.26	0.105	0.126	0.126	0.132	0.068	0.068	0.715	0.715	0.95
rs7526293	152,710,833	3' downstream	0.022	0.021	0.003	0.228	0.068	0.256	0.256	0.111	0.134	0.134	0.139	0.06	0.06	0.688	0.688	0.91

IL-6=interleukin-6; IL-6R=IL-6 receptor; SNP=single nucleotide polymorphism.

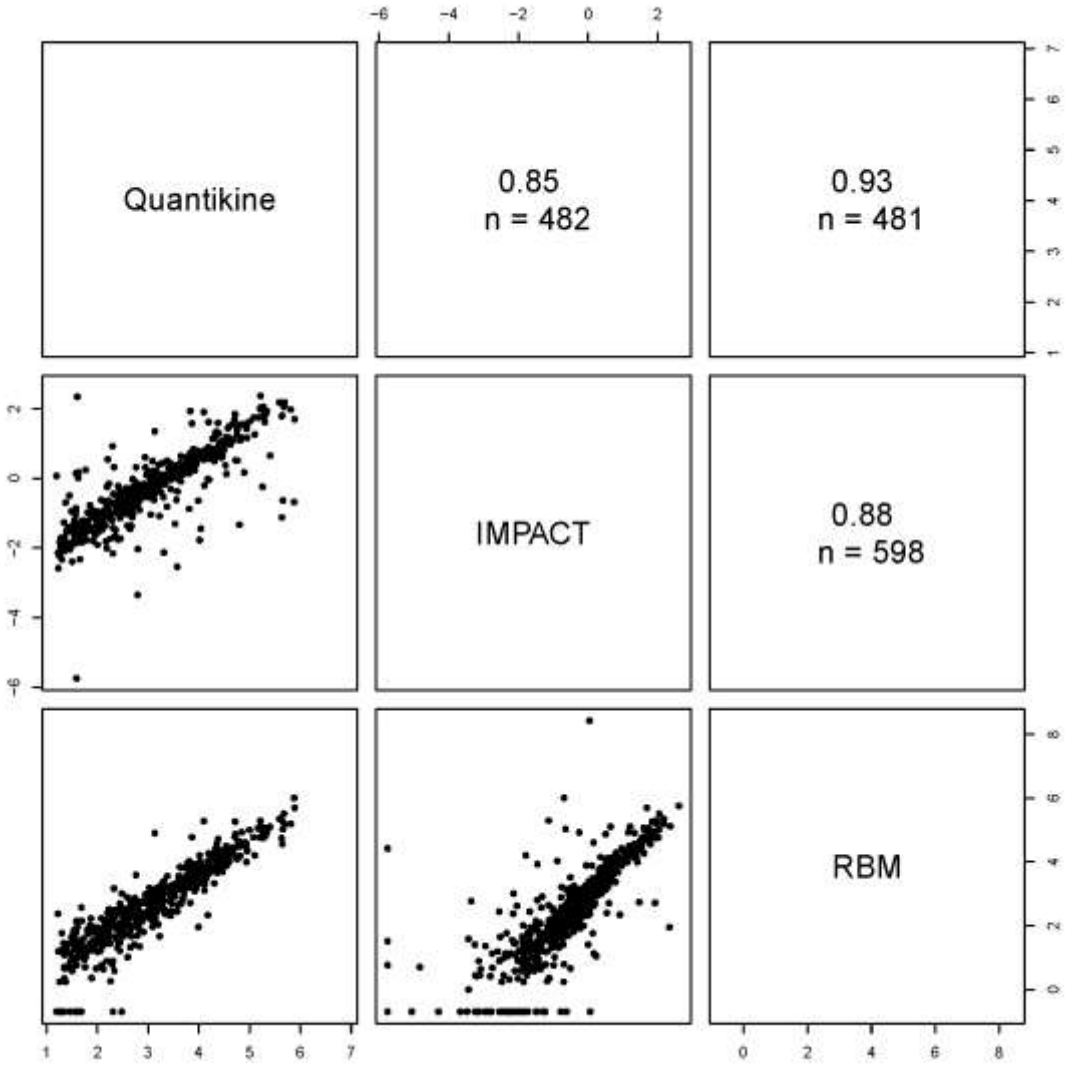
Supplementary Table 2. Association of baseline markers with treatment response measured by change in DAS28 from baseline at week 16.

Serum protein markers							
		Pooled DMARD-IR		**AMBITION**		**RADIATE**	
		TCZ	**Placebo**	**TCZ**	**Placebo**	**TCZ**	**Placebo**
IL-6	n	1547	781	207	204	214	116
	β	–0.17	0.12	–0.22	0.07	–0.16	0.22
	SE	0.04	0.05	0.11	0.11	0.10	0.13
	p	<0.0001	0.011	0.04	0.49	0.12	0.08
sIL-6R	n	1772	897	247	250	287	144
	β	0.04	0.01	0.03	–0.01	0.11	–0.04
	SE	0.03	0.05	0.13	0.14	0.11	0.14
	p	0.19	0.77	0.80	0.97	0.32	0.76

Primary RNA markers					
			RADIATE		
Gene	**Probe set**		**TCZ 8 mg/kg**	**TCZ 4 mg/kg**	**Placebo**
IL-6R	205945_at	n	76	75	66
		β	–0.1	0.06	–0.03
		SE	0.17	0.15	0.17
		p	0.53	0.68	0.86
	217489_s_at	n	76	75	66
		β	–0.14	0.06	0.09
		SE	0.19	0.14	0.14
		p	0.47	0.68	0.53
	226333_at	n	76	75	66
		β	0.03	0.11	0.28
		SE	0.19	0.12	0.17
		p	0.87	0.39	0.12

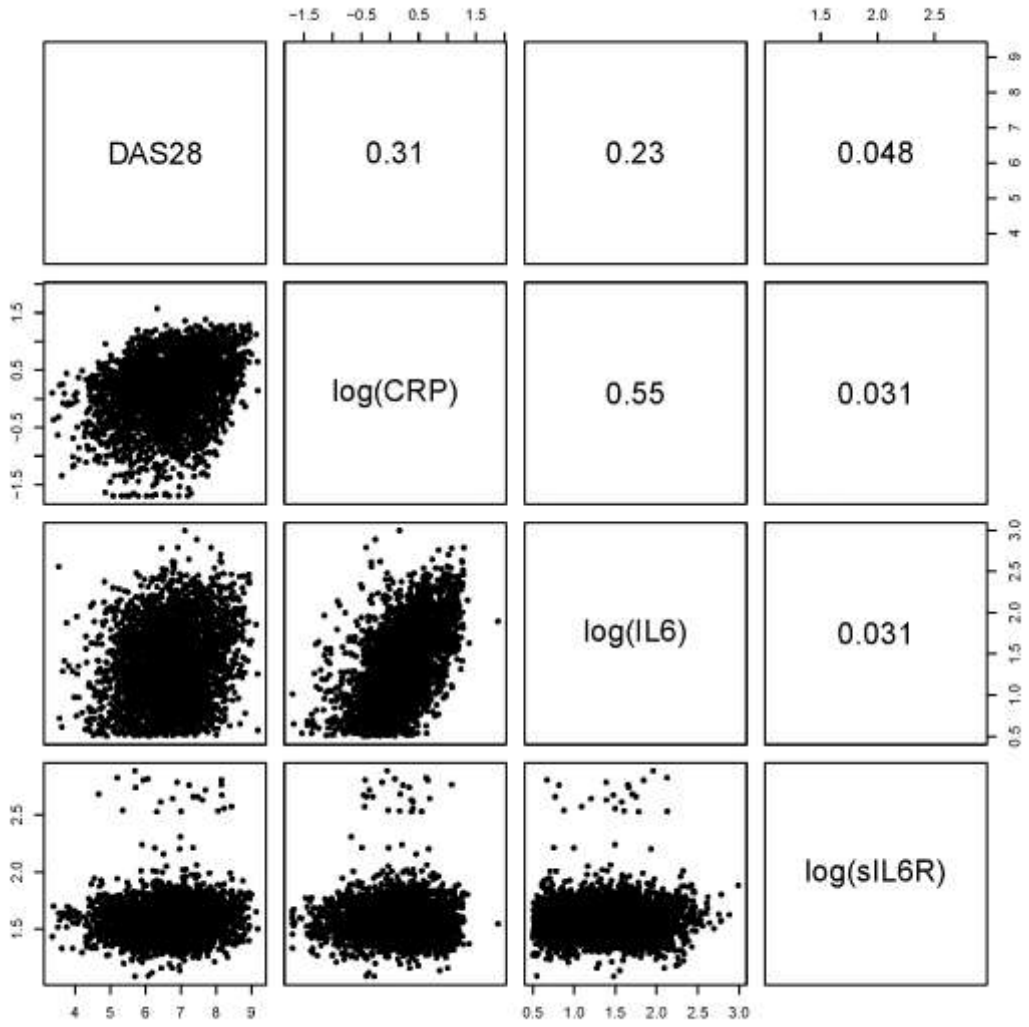
β=regression coefficient, representing the difference in DAS28 corresponding to each copy of minor allele; DAS28=Disease Activity Score using 28 joints; DMARD=disease-modifying anti-rheumatic drug; IL-6R=interleukin-6 receptor; IL-6ST=IL-6 signal transducer; IR=inadequate responder; sIL-6R=soluble IL-6R; TCZ=tocilizumab.
Baseline DAS28 and TCZ dose were included in the model as covariates. Among the seven-probe set in IL-6ST, no association was found. All estimated β values were <0.3

Supplementary Figure 1. Scatter plots showing correlations between the variables.
(A) Pair-wise scatter plot of the three serum IL-6 assays on baseline samples in the TOWARD study. Spearman rank correlation coefficient and sample size are shown in the upper panel.



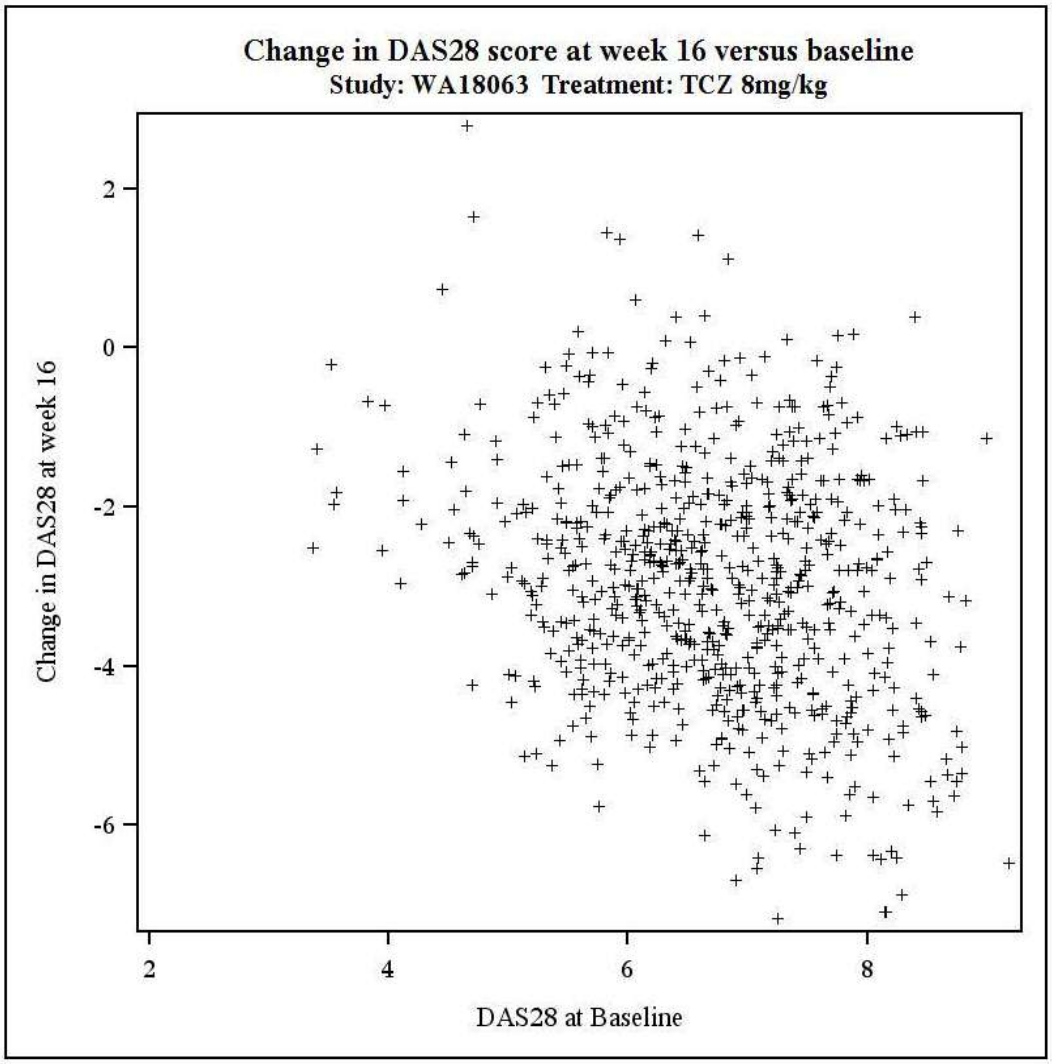
IL-6=interleukin-6; RMB=Rules-Based Medicine assay.

(B) Pair-wise scatter plots between DAS28, CRP, serum IL-6, and serum sIL-6R. Pearson correlation coefficients are labelled in the upper panel.



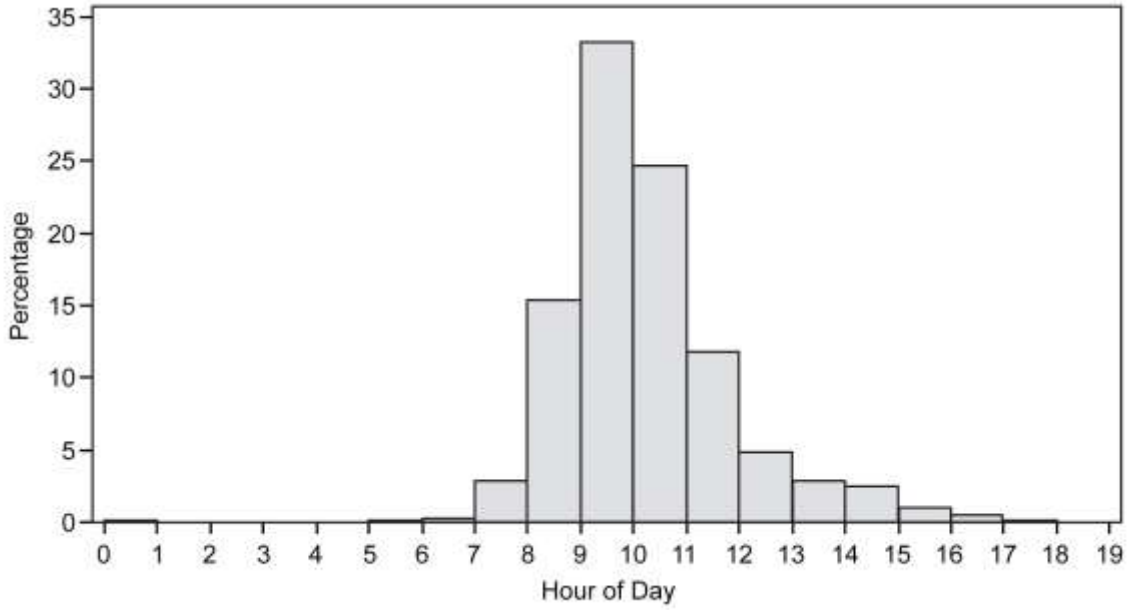
CRP=C-reactive protein; DAS28=Disease Activity Score at 28 joints; IL-6=interleukin-6; IL-6R=interleukin-6 receptor; sIL-6R=soluble interleukin-6 receptor.

(C) Scatter plot of change in DAS at week 16 versus baseline in tocilizumab-treated patients in the TOWARD study.

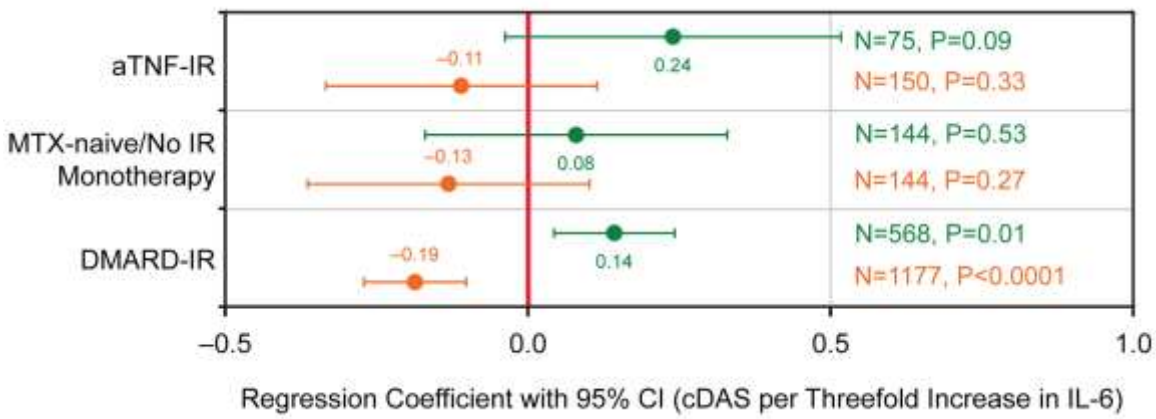


DAS=Disease Activity Score; DAS28=Disease Activity Score at 28 joints.

Supplementary Figure 2. Sampling time (hour of day) for baseline IL-6 samples in all studies (N = 3,143).



Supplementary Figure 3. Association of serum IL-6 concentration with response to treatment in patients who were rheumatoid factor positive at baseline.



•, MTX/DMARD; •, TCZ+MTX/DMARD.
Forest plots show the effect and 95% CI for the association of IL-6 with cDAS28 at 16 weeks across treatment lines.
cDAS28, change in DAS28 from baseline at week 16; CI, confidence interval; DMARD, disease-modifying antirheumatic drug; IL-6, interleukin-6; MTX, methotrexate; SD, standard deviation; TCZ, tocilizumab; TNF, tumour necrosis factor.

IL-6 pathway–driven investigation of response to IL-6 receptor inhibition in rheumatoid arthritis

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Clinical Trials Information

Registration names and numbers of tocilizumab clinical trials on Clinicaltrials.gov are OPTION NCT00106548, TOWARD NCT00106574, RADIATE NCT00106522, AMBITION NCT00109408, LITHE NCT00106535 and MEASURE NCT00535782.

ABSTRACT (261 words; maximum, 300)

Objectives To determine whether heterogeneity in interleukin-6 (IL-6), IL-6 receptor and other components of the IL-6 signalling pathway/network, at the gene, transcript and protein levels, correlates with disease activity in patients with rheumatoid arthritis (RA) and with clinical response to tocilizumab.

Design Biomarker samples and clinical data for five phase 3 trials of tocilizumab were analysed using serum (3,751 samples), genotype (927 samples) and transcript (217 samples) analyses. Linear regression was then used to assess the association between these markers and either baseline disease activity or treatment response.

Results Higher baseline serum IL-6 levels were significantly associated (P<0.0001) with higher baseline DAS28, ESR, CRP and HAQ in patients who had inadequate responses to disease-modifying anti-rheumatic drugs (DMARD-IR), patients who had inadequate responses to anti-tumour necrosis factor (aTNF-IR) and patients who were naive/responders to MTX. Higher baseline serum IL-6 levels were also significantly associated with better clinical response to tocilizumab (versus placebo) measured by cDAS28 in the pooled DMARD-IR (P<0.0001) and MTX-naive populations (P=0.04). However, the association with treatment response was weak. A threefold difference in baseline IL-6 level corresponded to only a 0.17-unit difference in DAS28 at week 16. IL-6 pathway SNPs and RNA levels also were not strongly associated with treatment response.

Conclusions Our analyses illustrate that the biological activity of a disease-associated molecular pathway may impact the benefit of a therapy targeting that pathway. However, the variation in pathway activity, as measured in blood, may not be a strong predictor.

These data suggest that the major contribution to variability in clinical responsiveness to therapeutics in RA remains unknown.

Article Summary

Article focus

- This study aimed to determine whether genetic polymorphism and heterogeneity in expression of IL-6 pathway/network components could explain the variability in baseline disease activity and clinical response to tocilizumab in patients with RA

Key messages

- Higher baseline serum IL-6 levels were significantly associated with higher baseline disease activity (ESR, CRP, DAS28 and HAQ scores) in large populations of methotrexate-naive, DMARD-IR and aTNF-IR patients with RA. Higher baseline serum IL-6 levels also showed significant correlation with better clinical response to tocilizumab (cDAS28) in DMARD-IR and MTX-naive RA patients; however, the effect size was small, limiting the clinical usefulness of the marker in predicting treatment benefit. A threefold higher baseline IL-6 concentration corresponded to only an additional 0.17-unit decrease in DAS28 score in the DMARD-IR patients treated with tocilizumab
- None of the genetic polymorphisms in IL-6 or IL-6R showed any association with treatment response to tocilizumab
- No strong predictor of tocilizumab treatment response was identified; thus, the major factors underlying the patient-to-patient variability in clinical

responsiveness to RA therapies remains unknown. This study illustrated well the difficulty of identifying clinically useful predictive biomarkers in a complex chronic disease

Strengths and limitations of this study

- This study included well-characterised RA patients from five large phase 3 controlled clinical trials. Biomarker data were available for more than 3,700 patients. The extensive collection of RNA, DNA and serum samples allowed thorough investigation of the target-related heterogeneity
- This is the first demonstration of a significant association between baseline serum IL-6 levels and baseline disease activity in a large population of patients with RA, extending earlier findings of elevated serum IL-6 levels in RA patients compared with healthy controls
- The current biomarker analysis is focused on clinical usefulness and included only blood samples. Any potentially useful information about local regulation of IL-6 pathway gene transcription and protein level in tissues involved in RA could not be obtained

INTRODUCTION

Interleukin-6 (IL-6) is a pleiotropic cytokine important in autoimmune diseases, including rheumatoid arthritis (RA).[1] High concentrations of IL-6 are found in the synovial fluid and sera of patients with RA. IL-6 involvement in RA pathogenesis includes involvement in B-cell proliferation and cytokine production, matrix metalloproteinase expression, acute-phase response and anaemia.[2]

IL-6 signal transduction occurs through either a classical (*cis*-) or a *trans*-signalling pathway. In *cis*-signalling, IL-6 binds to membrane-bound IL-6 receptor alpha (mIL-6Rα) before forming a trimer with gp130 (encoded by IL-6 signal transducer [*IL-6ST*]). A signalling complex is formed when this heterotrimer forms a dimer with another IL-6/mIL-6R/gp130 complex.[1] However, though gp130 is ubiquitously expressed, mIL-6R expression is restricted to hepatocytes and a subset of leukocytes. The *trans*-signalling pathway makes use of a soluble form of IL-6R (sIL-6R), to which IL-6 binds before forming a heterodimer with membrane-bound gp130, allowing cells that do not express mIL-6R to be activated by IL-6.[1]

Studies of tocilizumab,[3-8] a humanised monoclonal antibody targeted to IL-6R (membrane and soluble) that inhibits the interaction of IL-6 with its receptor, have shown meaningful amelioration of RA. However, the response is heterogeneous, consistent with the responses of other RA therapies. In a pooled analysis of patients who were inadequate responders to disease-modifying anti-rheumatic drug (DMARD-IR) in pivotal trials, 39% of those receiving 8 mg/kg tocilizumab for 24 weeks failed to achieve American College

of Rheumatology (ACR) 20 response (a 20% improvement), whereas 18% did achieve ACR70 response (a 70% improvement). Therefore, there is great interest in discovering biomarkers to aid in physician decision-making by the accurate prediction of clinical response in individual patients.

IL-6 and IL-6R levels vary in the sera of RA patients.[9,10] It has been suggested that some of this heterogeneity is genetically determined. The rs8192284 A/C polymorphism is found at the cleavage site of *mIL-6R* (Gln 357/Asp358) and has been associated with increased sIL-6R levels and RA susceptibility,[11,12] whereas a polymorphism at –174 in the promoter region of *IL-6* (rs1800795) affects transcription activity and, thus, serum levels of IL-6.[13] Beyond RA, IL-6R polymorphisms that reduce cardiovascular risk[14,15] attenuate classical IL-6 signalling by increasing the cleavage rate of mIL-6R (rs2228145 and rs8192284) and mirror the effects of tocilizumab on levels of IL-6 (increase), C-reactive protein (CRP) and fibrinogen (decrease). In asthma, the rs4129267 polymorphism in IL-6R was found to be associated with increased risk, and Ferreira et al[16] suggested that an IL-6R antagonist may show efficacy in asthma in a genotype-dependent manner. The aim of this study was to determine whether baseline heterogeneity in IL-6, IL-6R and other components of the IL-6 signalling pathway/network, at the gene, transcript and protein levels, correlates with disease activity in RA and clinical response to tocilizumab.

METHODS

Patients and samples

Serum specimens used in this study were collected from five pivotal phase 3 studies of tocilizumab in patients with RA: RADIATE,[4] OPTION,[3] TOWARD,[5] AMBITION[6] and LITHE[7] (Table 1). At baseline, serum samples were taken pre-dose and at fasting per protocol, resulting in the vast majority of them collected in the morning. The distribution of the sampling times is shown in Supplementary Figure 2. DNA samples were from RADIATE, OPTION and TOWARD, and RNA samples were from RADIATE. These trials evaluated tocilizumab (4 mg/kg or 8 mg/kg), administered every 4 weeks over a period of 24 weeks or longer, compared with placebo. The study populations differed according to background therapy with methotrexate (MTX) (OPTION, LITHE, RADIATE), any DMARD (TOWARD) or no DMARD (AMBITION). They also differed according to previous inadequate response to anti-tumour necrosis factor (aTNF) agents (RADIATE), MTX (OPTION, TOWARD, LITHE) or neither (AMBITION). In all studies, oral corticosteroids (≤10 mg/day prednisone or equivalent) were permitted if the dose was stable for at least 6 weeks before baseline. The study protocols were approved by relevant institutional review boards or ethics committees, and written informed consent was obtained from each patient. The studies were carried out in full concordance with the principles of the Declaration of Helsinki and with the laws and regulations of the country in which the research was conducted. DNA and RNA samples were collected only from patients who gave separate informed consent. DNA and RNA extraction methods are described in the Supplementary Material.

Serum analysis for IL-6 and sIL-6R

Three assays for serum IL-6 levels were used to analyse samples from TOWARD, as follows: Quantikine Human IL-6 Immunoassay (R&D Systems Inc., Minneapolis, MN), performed by Huntington Life Sciences (Huntington, UK) according to the instructions of the manufacturer (Roche Diagnostics, Penzberg, Germany) of the IL-6 assay[17]; IMPACT platform,[18] performed by Roche Diagnostics; and RBM IL-6 assay, performed by Myriad RBM (Austin, TX). Given the tight correlations among these three assays (Supplementary Figure 1A), we chose to analyse data from the Quantikine Human IL-6 Immunoassay (R&D Systems Inc.) because they were available for nearly all patients in all five core studies. The limit of detection of this IL-6 assay is 3.12 pg/ml. The assay is unaffected by the addition of recombinant human sIL-6R[19]; therefore, it can be assumed that the data reflect the total concentration of IL-6 (i.e. the concentration of free IL-6 plus the amount of IL-6 bound to sIL-6R).

Serum concentrations of endogenous sIL-6R were measured by Huntington Life Sciences using the Quantikine Human sIL-6R Immunoassay (R&D Systems Inc.) according to the manufacturer's instructions. The sensitivity of the sIL-6R assay is 31.2 pg/ml. Measured sIL-6R concentrations are insensitive to the addition of recombinant human IL-6 but are sensitive to the presence of tocilizumab.[20] Therefore, it can be assumed that the sIL-6R data presented reflect the total amount of free sIL-6R plus sIL-6R complexed with IL-6 plus (in samples that contain tocilizumab) an unquantified fraction of sIL-6R bound by tocilizumab.

Genotyping

DNA samples were genotyped using HumanHap550k BeadChip version 3.0 (Illumina, San Diego, CA) and custom chips using the OPA genotyping assay. Quality control (QC) methods for DNA samples and genotyping data are described in the Supplementary Material. High-quality markers with call rates ≥95% were included in the analysis. To reduce the multiplicity of hypothesis testing, a targeted approach was taken. Three groups of genes were considered in this analysis. Tier 1 markers consisted of 26 polymorphisms within the *IL-6* and *IL-6R* regions (Supplementary Table 1). Ten SNPs were selected in the IL-6 region, which is defined as 5 kb upstream and 5 kb downstream of the transcription coordinate; the SNPs cover a region of 12.6 kb. Similarly, 18 SNPs were selected in the IL-6R region, covering a region of 62.2 kb. The association of these markers with treatment benefit represents primary hypotheses. Tier 2 markers consisted of 233 polymorphisms within 22 genes identified as in the IL-6 pathway, termed the IL-6 canonical pathway (Supplementary Material). Tier 3 markers consisted of 1,005 polymorphisms in 67 genes, identified with GeneGo (Carlsbad, CA) software, related to IL-6 signalling, herein termed the IL-6 network (Supplementary Material). Markers for tiers 2 and 3 correspond to the decreasing strength of the hypothesis and the increasing dimensions compared with tier 1 markers. This approach provides greater power in the confirmatory analysis for the primary biomarker hypothesis yet allows for focused exploratory analysis to address the secondary hypotheses.

Polymerase chain reaction (PCR)–based HLA-DRB1 typing was performed on the DNA samples independently of the genotyping using whole genome and custom arrays. The

number of shared epitope alleles (0, 1 or 2) was determined for each patient based on his or her HLA-DRB1 genotype.

Transcript analysis

Two hundred thirty-three RNA samples, prepared from whole blood, at baseline were analysed using the GeneChip® Human Genome U133 Plus 2.0 Array (Affymetrix, Santa Clara, CA). After samples that failed quality control tests were removed (Supplementary Material), data for the remaining 217 samples were normalised using the Robust Multi-Array Analysis normalisation[21] (Bioconductor). Transcripts for *IL-6*, *IL-6R* and *IL-6ST* were extracted from the normalised data. IL-6 transcript levels were not analysed because of the low levels of signal observed. Three probe sets targeting *IL-6R* were analysed. Of those, 205945_at and 217489_at expression levels were found to be strongly correlated (Pearson's r^2=0.75), and the third probe set, 226333_at, had a weaker correlation with the other two (Pearson's r^2=0.34 and r^2=0.38). These three probe sets represent the primary hypothesis in RNA transcripts. In addition, seven probe sets from *IL-6ST* were analysed as secondary markers.

Measures of disease activity and clinical response

Six different measures were used for disease activity at baseline: erythrocyte sedimentation rate (ESR), CRP, swollen joint count at 28 joints (SJC28), tender joint count at 28 joints (TJC28) and Health Assessment Questionnaire (HAQ), with disease activity at 28 joints (DAS28; derived from the four core components of ESR, SJC, TJC and patient global assessment) considered the primary end point. As a measurement of

treatment response, change in DAS28 from baseline at week 16 (cDAS28) was mainly used. Unlike dichotomous responder criteria (e.g. ACR response, DAS28 remission and clinical disease activity index remission), this continuous measure captures the range of individual responses and is therefore more sensitive in detecting the effect of biomarkers across different levels of prevalence. In all studies except AMBITION, patients were allowed to enter escape therapy at 16 weeks if they had inadequate responses. Therefore, cDAS28 was used at week 16 to increase the sample size and to minimise bias.

Statistical analysis

Table 2 provides a summary of all markers analysed. Quality control analyses were performed for genotyping and gene expression data, as described in the Supplementary Material; assays and samples with poor data quality were removed. Distribution was assessed for each marker, and appropriate transformation was applied before further analysis. Minor allele frequencies and Hardy-Weinberg equilibrium were calculated for single nucleotide polymorphisms (SNPs). Given that the distribution within each gene expression probe set was approximately normal, no further transformation was applied. Log transformation was applied to the protein markers IL-6 and sIL-6R because of the skewed distribution of raw values.

Correlations among the baseline markers were assessed within each sample type to enable understanding of the amount of information shared among the assays. For SNPs, linkage disequilibrium coefficients within IL-6 and IL-6R were obtained from HapMap 22 based on the CEU (Utah residents of Northern and Western European ancestry from

the CEPH collection) data. For the gene expression markers, correlation coefficients were calculated among probe sets within *IL-6R* and *IL-6ST*. For the proteins, correlation was calculated among the three assays for IL-6 and between IL-6 and sIL-6R. Pearson or Spearman rank correlation was used as appropriate.

Linear regression was used to assess the association between markers with baseline disease activity and markers with treatment response. Because of the skewed distribution of the raw values, log(ESR) and log(CRP+1) were used in the regression analysis. All patients were included in the regression analysis of baseline disease activity. In the regression analysis of cDAS28, separate analyses were undertaken in patients treated with tocilizumab and those administered placebo. Given that baseline DAS28 is strongly associated with change in DAS28 at week 16, it was included as a covariate in all models. No additional covariates were included in the model. In addition, the number of shared epitope alleles[22] (0, 1 or 2) and the tocilizumab dose were used as covariates in the analysis of genetic data.

The numbers of genetic markers tested were 18, 233 and 1,004, respectively, for the tier 1, 2 and 3 markers. In addition, six baseline end points were used on the baseline disease association analysis. Analyses were undertaken in the white population and the all-patient population. To adjust for multiple testing, the false discovery rate (FDR)[23] was calculated within each tier and within the white population and the all-patient population. This FDR accounted for the multiplicity of markers and the end points.

To help interpret the outcome of the regression analysis and to enable direct comparison of the analysis results across all markers, consistent representations of the results were used. For continuous markers, the regression coefficients were standardised to reflect how many units of change in the end points corresponded to a difference of 1 standard deviation (SD) in the marker value. For the SNP markers, an allelic model was used (i.e. two copies of the minor allele had twice the effect of one copy). The regression coefficient corresponded to the change in end point per copy of minor allele.

RESULTS

Baseline characteristics of patients with DNA, RNA and serum samples

Baseline characteristics of the patient subgroups with DNA, RNA and serum samples available are shown in Table 1. They are generally comparable to the overall population for each study. Patient ethnicity is relevant to the genetic analysis. Therefore, the proportion of patients of European ancestry (White) is shown for the DNA subpopulations. Of the 927 patients with DNA samples, 730 (79%) were of European ancestry (White) (Table 1). As expected based on treatment experience, patients from the MTX-naive or the MTX-free study had shorter disease duration, and those from the aTNF-IR study had longer disease duration. For serum markers where large sample size was available, data were analysed separately for the MTX-naive/MTX-free population, pooled DMARD-IR population and aTNF-IR population. The distribution of baseline IL-6 was similar in the rheumatoid factor–positive and –negative subpopulations (45.2 ± 60.5 and 41.5 ± 60.6 [mean ± SD], respectively).

Associations with baseline disease activity

Higher baseline serum IL-6 levels were significantly associated with higher baseline DAS28, ESR, CRP and HAQ in all three populations analysed, with P<0.0001 in most cases (Table 3). The strongest association was observed between CRP and IL-6, for a correlation coefficient of 0.29 to 0.36 in three populations (Supplementary Figure 1B). IL-6 was not strongly associated with SJC28 or TJC28. Baseline sIL-6R was not strongly associated with any baseline disease activity measures.

Among the baseline RNA transcripts, an association was observed between the IL-6R transcript and CRP and ESR at baseline (data not shown). No association was found with the other baseline disease activity measures. Association with CRP and ESR was also observed, to a lesser degree, for an IL-6ST transcript.

After correction for multiple testing, no significant associations were found for any of the *IL-6* or *IL-6R* polymorphisms genotyped with baseline disease activity. Of the 1,237 polymorphisms representing 22 genes in the IL-6 canonical pathway, 67 additional genes in the IL-6 network, six baseline disease activity end points and two patient populations (all-patient, white), six associations were found to have borderline significance after adjusting for multiple testing, with an FDR of approximately 5%. There were SNPs in *TOLLIP* and *RUNX1* versus ESR and SNPs in *IL1RAPL1* versus SJC and ESR. Such borderline significance meant the association should be treated as a weak hypothesis.

Associations with clinical response to tocilizumab

Higher baseline serum IL-6 concentrations were significantly associated with better clinical response to tocilizumab but not to placebo, as measured by cDAS28 in the pooled DMARD-IR population (P<0.0001) (Supplementary Table 2, Figure 1). The same effect was observed in the MTX-naive population (P=0.04). In the aTNF-IR population, the association was not significant, partially because of the smaller sample size. In the DMARD-IR population, a threefold difference in baseline IL-6 concentration, equivalent to 1 SD in the baseline log(IL-6) distribution, corresponded to a small difference of 0.17 units in cDAS28 score at week 16. The association between serum IL-6 level and achievement of DAS28 remission (DAS28 <2.6) was analysed using receiver operating characteristic analysis in the pooled DMARD-IR population treated with tocilizumab (n=1,547). The area under the curve was only 0.59, suggesting that baseline serum IL-6 levels provided very little discrimination between those achieving and those not achieving DAS28 remission. In the placebo group, the direction of association was opposite that of tocilizumab-treated patients, indicating that baseline serum IL-6 level (with no longitudinal data on change in level) is not a prognostic factor for clinical response in RA in these cohorts.

Baseline sIL-6R concentration in serum had no effect on treatment response in either the tocilizumab or the placebo group (Supplementary Table 2). IL-6R whole blood mRNA (in RADIATE) was not associated with clinical response to tocilizumab or placebo treatment (Supplementary Table 2). No *IL-6* or *IL-6R* polymorphisms were associated with cDAS28 after correction for multiple testing. Although rs2069840 and rs12700386

had a raw P<0.05 (Table 4), the estimated effect size was small. Each copy of the minor allele for rs2069840 corresponded to only a 0.3-unit difference in cDAS28. Of the SNPs in the canonical and the network or signalling pathway, rs973767 (an intronic SNP in *PTPN2*) had the lowest P value (5.7×10^{-5}) but was not significant after correction for multiple testing (FDR=0.057; Table 4). In the rheumatoid factor–positive subpopulation, the effect of baseline serum IL-6 on clinical response was very similar to that in the overall population (Supplementary Figure 3).

DISCUSSION

We have investigated the associations among a number of DNA, RNA and protein biomarkers directly related to IL-6 signalling (Table 2) with baseline disease activity and treatment benefit from tocilizumab (Table 1). We demonstrate the modest association between serum IL-6 levels and clinical disease activity as measured by DAS28 and HAQ. Although serum IL-6 levels have previously been shown to be elevated in patients with RA compared with controls,[9,24] this is the first time a significant correlation between IL-6 level and disease activity has been demonstrated in a large population. IL-6 level accounted for only a small component of observed variance in disease activity (Supplementary Table 2, Figure 1). Of the four DAS28 components, serum IL-6 level was most strongly associated with ESR/CRP. This is not surprising given the pivotal role of IL-6 (and the pronounced inhibitory effect of tocilizumab) on the acute-phase response.[25] Interestingly, HAQ, a measure of patient-reported disability independent of inflammation, also had a significant association with serum IL-6 level.

Littman[26] proposes that IL-6/tocilizumab may represent 'low-hanging fruit' in terms of personalised health care in RA, but our analysis suggested otherwise. Higher baseline DAS28 was associated with higher change in DAS28 in response to therapy (Supplementary Figure 1C). When change in DAS28 was corrected for baseline DAS28, only a weak association with serum IL-6 was observed. Even though IL-6 has been shown to decrease with effective treatments, such as modified-release prednisone,[27] in RA, we saw no evidence that a single baseline 'snapshot' serum IL-6 level was a general prognostic factor for clinical response in RA (i.e. predicted subsequent treatment response or disease course).

Serum IL-6 exhibits a pronounced circadian rhythm.[28] This source of variability was minimised, but not eliminated, by collecting the samples in as narrow a time window as achievable in large clinical studies. It is possible that discordance in IL-6 signalling activity between blood and synovial tissue explains the lack of association with blood samples in our study. However, this cannot explain the negative genetic data. The lack of genetic associations between polymorphisms in *IL-6R* (including those determining sIL-6R levels) and baseline disease activity[11,29] shows that sIL-6R levels, though highly variable, are not appreciably different between patients who have RA and those who are healthy.[30,31] The effect of a genetic marker can be affected by a patient's genetic background. An ethnically homogeneous population is ideal for genetic analysis. In this cohort, 79% of the patients were of European ancestry (White). All genetic analyses were performed in the overall population for maximum sample size and in the White

subpopulation for genetic homogeneity. The White subpopulation was sufficiently large; therefore, the lack of a strong genetic effect is compelling.

Formal multiplicity adjustment was not carried out in the analysis of serum and RNA markers. However, informal assessment was used to help interpret the results of the statistical analysis. Multiplicity of the statistical testing was indicated by multiple markers and multiple end points; the numbers of markers in each sample type are shown in Table 2. Six baseline disease characteristics and one clinical response end point were used. In addition, genetic analyses were performed in the White and the overall populations. Overall, ≥10 tests were carried out for each serum and RNA marker; results with $P<0.0001$ would have been significant had adjustment for multiplicity been performed, and results with unadjusted $P>0.01$ can be regarded as likely false positives.

Power assessment was not performed. Empirically, given the large sample size for DNA and serum samples, the power to detect a clinically relevant effect at $\alpha=0.05$ was very high. Therefore, $P>0.05$ can be interpreted as evidence of no effect. Analyses of RNA markers had low power because of the limited sample size.

Our analyses illustrate that in a disease such as RA, variation in the abundance of a therapeutic target may not be a strong predictor of patient benefit. In particular, the response to tocilizumab was not dependent on IL-6R SNPs, which were found to be associated with asthma and cardiovascular risk. In addition, a genome-wide association approach using 1,600 patients from five controlled clinical trials did not yield any strong

predictor to tocilizumab treatment response.[32] These data suggest that the major contribution to variability in clinical responsiveness to therapeutics in RA remains unknown, and they illustrate the challenges of identifying predictive biomarkers, even in large, well-conducted studies.

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Competing interests

All authors are (or were at the time of the study) employees of Roche or Genentech, a member of the Roche group, and four authors (AH, AK, OH and RU) also own stock in Roche.

Author contributions

JW designed the study, conducted research, conducted the literature search, analysed and interpreted the data and drafted the manuscript; AP designed the study, conducted research, conducted the literature search, collected data, analysed and interpreted the data, oversaw data collection and drafted the manuscript; RU analysed and interpreted the data

and drafted the manuscript; SG designed the study and collected data; GL analysed and interpreted the data; CR analysed and interpreted the data; RB designed the study, collected data and analysed the data; AK analysed and interpreted the data; AH designed the study and collected data; MM designed the study; OH interpreted the data and drafted the manuscript.

All authors revised the manuscript critically for important intellectual content and approved the final version for submission.

Ethics approval

The study protocols were approved by relevant institutional review boards or ethics committees, and written informed consent was obtained from each patient. The studies were carried out in full concordance with the principles of the Declaration of Helsinki and with the laws and regulations of the country in which the research was conducted. DNA and RNA samples were collected only from patients who gave separate informed consent.

Data sharing

No additional data are available at this time.

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TABLES

Table 1. Demographics of patients from whom biological samples were collected and analysed and whose data passed a quality control check

Sample type	DNA			RNA	Serum				
Study	**OPTION**	**RADIATE**	**TOWARD**	**RADIATE**	**OPTION**	**LITHE**	**TOWARD**	**AMBITION**	**RADIATE**
Patient population	MTX-IR	aTNF-IR	DMARD-IR	aTNF-IR	MTX-IR	MTX-IR	DMARD-IR	MTX naive or free	aTNF-IR
Treatment	8 mg/kg TCZ + MTX	8 mg/kg TCZ + MTX	8 mg/kg TCZ + DMARD	8 mg/kg TCZ + MTX	8 mg/kg TCZ + MTX	8 mg/kg TCZ + MTX	8 mg/kg TCZ + DMARD	8 mg/kg TCZ + MTX	8 mg/kg TCZ + MTX
	4 mg/kg TCZ + MTX	4 mg/kg TCZ + MTX		4 mg/kg TCZ + MTX	4 mg/kg TCZ + MTX	4 mg/kg TCZ + MTX			4 mg/kg TCZ + MTX
	MTX	MTX	DMARD	MTX	MTX	MTX	DMARD	MTX	MTX
Total patients, n	286	178	463	217	603	1126	1010	581	431
TCZ, n	189	119	301	151	409	753	661	244	289
MTX, n	97	59	162	66	194	373	349	337	142
Age, years	51.3 (11.1)	51.5 (12.4)	54.2 (12.6)	53.2 (11.9)	51.0 (12.2)	51.9 (12.4)	53.4 (12.9)	50.3 (13.0)	52.9 (12.6)
Female, %	79.0	79.8	81.0	82.40	81.8	82.9	81.7	80.4	81.4
Height, cm	163 (9)	165 (8)	164 (9)	165 (8)	162 (9)	162 (9)	163 (9)	163 (9)	165 (8)
Weight, kg	73 (17)	77 (19)	76 (18)	77 (20)	79 (17)	73 (19)	74 (19)	73 (18)	75 (18)
RA duration, years	7.8 (7.3)	11.1 (8.3)	9.7 (9.1)	12.1 (9.6)	7.6 (7.3)	9.2 (8.0)	9.6 (8.8)	6.4 (8.2)	11.6 (9.1)
RF positive, %	76.2	79.8	75.6	76.5	77.6	82.1	77.1	72.5	75.2
White, %	73.8	93.3	76.2	Not applicable			Not applicable		
TCZ, n	141	109	233						
MTX, n	70	57	120						
DAS28	6.8 (0.9)	6.8 (1)	6.6 (1)	6.8 (0.9)	6.8 (0.9)	6.5 (0.9)	6.7 (1.0)	6.8 (0.9)	6.8 (1.0)
HAQ	1.6 (0.6)	1.7 (0.6)	1.5 (0.6)	1.7 (0.6)	1.6 (0.6)	1.5 (0.6)	1.5 (0.6)	1.5 (0.6)	1.7 (0.6)
SJC	20.9 (11.3)	18.8 (10.6)	20.5 (11.9)	18.1 (9.8)	20.0 (11.2)	16.9 (9.4)	19.6 (11.5)	19.8 (11.2)	18.9 (10.6)
TJC	32.3 (15.2)	31.2 (15.2)	30.1 (15.8)	31.4 (15.1)	32.6 (15.6)	28.3 (14.7)	30.0 (15.7)	31.9 (14.4)	31.1 (15.6)
CRP	2.4 (2.9)	3.7 (4.2)	2.3 (2.9)	3.3 (3.8)	2.6 (3.0)	2.2 (2.5)	2.6 (3.8)	3.0 (3.3)	3.2 (3.8)

aTNF, anti-tumour necrosis factor; CRP, C-reactive protein; DAS28 , Disease Activity Score using 28 joints; DMARD, disease-modifying antirheumatic drug; HAQ, Health Assessment Questionnaire; IR, inadequate responder; MTX, methotrexate; RA, rheumatoid arthritis; RF, rheumatoid factor; SJC, swollen joint count; TCZ, tocilizumab; TJC, tender joint count.

Values with numbers in parentheses are mean (SD).

Table 2. Summary of markers investigated. For the DNA markers, SNPs in IL-6 and IL-6R are considered tier 1, IL-6 network genes are considered tier 2, and IL-6 pathway genes are considered tier 3

Sample type	Target gene	Analytes, n	Assay (manufacturer)
Primary			
DNA	*IL-6*	10 SNPs	Bead-Chip arrays HumanHap550k version 3.0, OPA custom array (Illumina)
	IL-6R	18 SNPs	
RNA	*IL-6R*	3 probe sets	GeneChip Human Genome U133 Plus 2.0 Array (Affymetrix)
Serum	*IL-6*	1	Human IL-6 Immunoassay (Quantikine)
	sIL-6R	1	Human sIL-6R Immunoassay (Quantikine)
Secondary			
DNA	IL-6 network genes	233 SNPs (22 genes)	Bead-Chip arrays HumanHap550k version 3.0, OPA custom array (Illumina)
	IL-6 pathway genes	1004 SNPs (67 genes)	
RNA	*IL-6ST*	7 probe sets	GeneChip Human Genome U133 Plus 2.0 Array (Affymetrix)

IL-6, interleukin 6; IL-6R, IL-6 receptor; IL-6ST, IL-6 signal transducer; sIL-6R, soluble IL-6R; SNP, single-nucleotide polymorphism.

Table 3. Association of markers with baseline disease activity. All results for serum markers are shown. For RNA, the probe sets within each gene were strongly correlated. One probe set each in IL-6R and IL-6ST is shown. For DNA, only associations with raw P<0.01 (tier 1) and FDR P<0.05 (tiers 2 and 3) were shown. FDR was calculated within the population (white or all) and within the tier

Serum protein and RNA markers									
		Serum IL-6			Serum sIL-6R			IL-6R 217489_s_at	IL-6ST 234967_at
End point		AMBITION	RADIATE	Pooled DMARD-IR	AMBITION	RADIATE	Pooled DMARD-IR	RADIATE	RADIATE
DAS28	n	480	322	2286	577	423	2620	217	217
	β	0.209	0.257	0.201	–0.007	0.075	0.054	0.109	–0.04
	r^2	0.051	0.072	0.044	0	0.006	0.003	0.013	0.002
	p	<0.0001	<0.0001	<0.0001	0.8478	0.1018	0.0044	0.09	0.54
TJC	n	481	327	2305	580	428	2642	217	217
	β	–0.186	1.455	0.138	–0.154	1.28	0.054	0.27	1.276
	r^2	0	0.009	0	0	0.007	0	0	0.007
	p	0.7742	0.0938	0.6611	0.7967	0.0889	0.8558	0.79	0.2140
SJC	n	481	327	2305	580	428	2642	217	217
	β	0.247	1.421	0.813	–0.679	–-0.069	0.52	–0.864	0.218
	r^2	0	0.018	0.006	0.004	0	0.002	0.008	0
	p	0.6279	0.0158	0.0003	0.1424	0.8938	0.013	0.1950	0.7440
ESR	n	481	327	2299	580	428	2635	217	217
	β	0.19	0.172	0.151	0.039	–0.003	0.023	0.164	–0.132
	r^2	0.088	0.091	0.055	0.004	0	0.001	0.082	0.053

	p	<0.0001	<0.0001	<0.0001	0.132	0.9158	0.0672	<0.0001	0.0007
CRP	n	481	327	2305	580	428	2642	217	217
	β	0.363	0.429	0.35	0.007	0.019	0.019	0.228	–-0.145
	r^2	0.288	0.358	0.309	0	0.001	0.001	0.089	0.036
	p	<0.0001	<0.0001	<0.0001	0.8103	0.5935	0.1242	<0.0001	0.005
HAQ	n	480	324	2165	579	425	2465	217	217
	β	0.085	0.145	0.084	0.002	0.074	0.024	0.048	–0.103
	r^2	0.019	0.062	0.018	0	0.016	0.002	0.007	0.031
	p	0.0022	<0.0001	<0.0001	0.933	0.0089	0.0504	0.22	0.009

DNA markers

Tier	Gene	Chr	SNP	Population	MAF	End point	n	β	SE	Raw P	FDR P
1	*IL-6R*	1	rs1386821	White	0.17	HAQ	708	0.135	0.041	0.001	0.18
				All	0.15	HAQ	845	0.118	0.040	0.004	0.52
3	*TOLLIP*	11	rs5743899	All	0.22	ESR	893	0.15	0.03449	1.5E-05	0.046
	RUNX1	21	rs2252585	All	0.32	ESR	905	0.1209	0.02884	3.0E-05	0.046
	IL-1RAPL1	23	rs12559028	All	0.49	SJC	905	2.182	0.5349	4.9E-05	0.050
			rs5943618	All	0.44	SJC	905	2.27	0.5424	3.1E-05	0.046
			rs4829239	All	0.38	SJC	905	2.321	0.5592	3.6E-05	0.046
			rs5927671	All	0.45	ESR	897	0.1181	0.02853	3.8E-05	0.046

β, regression coefficient, representing the difference in the end point corresponding to standard deviation in marker value; Chr, chromosome; CRP, C-reactive protein; DAS28, Disease Activity Score using 28 joints; DMARD, disease-modifying antirheumatic drug; ESR, erythrocyte sedimentation rate; HAQ, Health Assessment Questionnaire; IL-6ST, IL-6 signal transducer; IR, inadequate responder; MAF, minor allele frequency; MTX, methotrexate; SJC, swollen joint count; TCZ, tocilizumab; TJC, tender joint count.

Table 4. Association of baseline markers with treatment response measured by change in DAS28 from baseline at week 16. Baseline DAS28 and TCZ dose were included in the model as covariates. FDR was calculated within the white or all population and within the tier

All association results for primary DNA markers											
Gene	SNP	All DMARD-IR, TCZ					White DMARD-IR, TCZ				
		MAF	n	β	SE	Raw P	MAF	n	β	SE	Raw P
IL-6	rs12700386	0.19	583	0.22	0.11	0.049	0.18	468	0.16	0.13	0.20
	rs2069833	0.35	584	–0.08	0.08	0.34	0.42	469	–0.13	0.09	0.18
	rs2069837	0.08	586	–0.11	0.15	0.44	0.08	471	–0.05	0.16	0.75
	rs2066992	0.12	586	–0.09	0.12	0.49	0.06	471	–0.11	0.21	0.61
	rs2069840	0.32	584	0.24	0.09	0.007	0.33	469	0.30	0.10	0.0026
	rs1554606	0.4	586	–0.10	0.08	0.23	0.45	471	–0.15	0.09	0.12
	rs2069845	0.4	583	–0.10	0.08	0.23	0.45	468	–0.15	0.09	0.12
	rs2069861	0.07	584	–0.10	0.15	0.53	0.08	469	–0.11	0.16	0.50
	rs10242595	0.36	586	–0.02	0.08	0.86	0.32	471	–0.02	0.10	0.80
	rs11766273	0.07	586	0.10	0.16	0.53	0.08	471	0.05	0.17	0.75
IL-6R	rs1386821	0.15	586	0.02	0.12	0.87	0.17	471	0.06	0.13	0.67
	rs4075015	0.39	584	–0.06	0.09	0.50	0.41	469	–0.07	0.10	0.46
	rs6684439	0.41	586	0.03	0.08	0.76	0.39	471	0.06	0.10	0.55
	rs4845618	0.43	582	0.03	0.08	0.74	0.44	467	0.00	0.09	0.99
	rs8192282	0.15	581	–0.09	0.12	0.46	0.16	468	–0.08	0.13	0.53
	rs7549250	0.43	575	0.07	0.08	0.43	0.44	462	0.02	0.09	0.81
	rs4553185	0.43	586	0.04	0.08	0.60	0.44	471	0.01	0.09	0.90
	rs4845623	0.44	586	–0.01	0.08	0.92	0.41	471	0.01	0.10	0.89

	rs4537545	0.44	586	–0.04	0.08	0.60	0.41	471	–0.01	0.10	0.93
	rs4129267	0.41	586	–0.02	0.08	0.77	0.4	471	0.02	0.10	0.87
	rs8192284	0.41	582	–0.03	0.08	0.76	0.4	467	0.02	0.10	0.87
	rs11265618	0.17	586	–0.05	0.11	0.64	0.17	471	–0.04	0.13	0.73
	rs4329505	0.16	582	–0.03	0.11	0.81	0.16	467	–0.02	0.13	0.87
	rs4240872	0.24	586	0.03	0.10	0.79	0.23	471	–0.01	0.11	0.93
	rs4509570	0.24	580	0.02	0.10	0.85	0.23	466	–0.02	0.11	0.83
	rs2229238	0.19	584	–0.03	0.11	0.75	0.19	469	0.00	0.12	0.98
	rs7514452	0.19	586	–0.03	0.11	0.77	0.19	471	0.00	0.12	0.98
	rs7526293	0.2	584	–0.04	0.10	0.71	0.2	469	–0.03	0.12	0.79

DNA markers analyzed in the pooled DMARD-IR population with an FDR* of <0.1

Tier	Gene	SNP	Population	MAF	n	β	SE	RAW P	FDR P
1	*IL-6*	rs2069840	White	0.33	469	0.30	0.10	0.0026	0.073
3	*PTPN2*	rs973767	All	0.14	586	0.48	0.12	7.2E-05	0.072
			White	0.16	471	0.53	0.13	5.7E-05	0.057

β, regression coefficient, representing the difference in DAS28 corresponding to each SD in marker value; DAS28, Disease Activity Score using 28 joints; DMARD, disease-modifying antirheumatic drug; FDR, false discovery rate; IL-6, interleukin-6; IL-6R, IL-6 receptor; IR, inadequate responder; MAF, minor allele frequency; SNP, single nucleotide polymorphism; TCZ , tocilizumab.

FIGURE

Figure 1. Serum IL-6 concentration association with response to treatment. Forest plots show the effect and 95% CI for the association of IL-6 with cDAS28 at 16 weeks across treatment lines. cDAS28, change in DAS28 from baseline at week 16; CI, confidence interval; DMARD, disease-modifying antirheumatic drug; IR, inadequate responder; MTX, methotrexate; SD, standard deviation; TCZ, tocilizumab; TNF, tumour necrosis factor. •, MTX/DMARD; •, TCZ+MTX/DMARD.