Potential role of pharmacogenetics for optimalization of drug therapy in rheumatoid arthritis

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Chapter 10:

Pharmacogenetic pathway approach detects associations with adalimumab efficacy in rheumatoid arthritis

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Abstract

Objective
Adalimumab efficacy in patients with RA is about 60-70% and predictors for response are yet unknown. This study aims at exploring predictors for adalimumab efficacy in RA patients by associating response with single nucleotide polymorphisms (SNPs) in: 1) the pharmacological pathway of adalimumab, and 2) disease susceptibility genes.

Methods
223 SNPs in 111 genes were analyzed in 325 genotyped rheumatoid arthritis (RA) patients treated with adalimumab. Treatment outcome was evaluated with the use of the 28-joint disease activity score criteria (DAS28) by the primary endpoints EULAR good response and remission and by the secondary endpoint relative change in DAS28. Initially SNPs were explored for associations under allelic and genotypic model using chi-square tests. Hereafter, SNPs were investigated in the most appropriate model with Cochrane-Armitage test for trend analysis and regression analyses under additive, recessive and dominant genetic model with the covariates age, gender, concomitant MTX therapy and DAS28 at baseline.

Results
19 SNPs, 11 SNPs and 8 SNPs were significantly associated with EULAR good response, EULAR remission and relative change in DAS28, respectively ($p<0.05$). Four SNPs, rs1126535 in *CD40LG*, rs682847 in *KDR*, rs1267067 in *TANK* and rs25648 in *VEGFA*, were significantly associated with adalimumab treatment outcome according to all three endpoints ($p<0.05$).

Conclusion
The SNPs, rs1126535 in *CD40LG*, rs6828477 in *KDR*, rs1267067 in *TANK* and rs25648 in *VEGFA* were found potential predictors for adalimumab efficacy. The results from this explorative study provide new insights regarding the potential of pharmacogenetics related to adalimumab efficacy in RA.

Introduction

TNF inhibitors have proven to be effective in a majority of rheumatoid arthritis (RA) patients, which had not experienced a positive response to traditional disease-modifying antirheumatic drugs (DMARDs) (1-3). Consequently, these biologicals have become important agents in therapy of severe RA. Still, a considerable proportion of 30 to 40% of these patients do not experience benefit from this treatment. If patients do not profit from TNF inhibitor therapy, disease progression and joint erosion proceeds while patients potentially experience serious drug related side effects. The idea of a priori prediction of drug response in RA patients is a highly relevant topic, as it will enable rheumatologists to readily identify those patients sensitive to certain drug regimens and thereby minimizing irreversible joint dysfunction existing in severe untreated RA (4-6).
Variation in treatment response for patients treated with TNF inhibitors could be expected, regarding interdifferential cytokine and receptor expression in patients leading to heterogeneity of cytokine presence and regulation in joints. Although this has not been studied intensively, indeed several polymorphisms have been studied over the years, which may associate with treatment outcome of TNF inhibitors in general (7-9).
It has been suggested that adalimumab and other TNF inhibitors target analogous functional pathways (10;11). Despite the awareness that the mechanism of action of TNF inhibitors may involve several pathways and is, therefore not monogenetic, so far, the majority of association studies have analyzed only genetic variants in single genes in relation with treatment outcome.
Alternatively, since a large group of enzymes and cytokines are likely involved in the pharmacodynamics of TNF inhibitors and inflammatory cascade of RA, an approach concerning reciprocal comparison of multiple genes encoding these proteins would be more logical. Characteristically, with this systems pharmacology approach one considers the variability in the entire pathway without restricting the analysis to only one candidate gene (12). Moreover, with the single candidate gene method, SNPs that are responsible for the rate limiting or extending step in mechanism of action are easily missed.
Therefore, the objective of this study is to explore whether the efficacy of treatment with adalimumab is associated with single nucleotide polymorphisms (SNPs) in genes related to the mechanism of action of TNF inhibitors and/or inflammatory process of RA by using a systems pharmacology approach. Similarly, SNPs, which were previously associated with genetic susceptibility to RA and/or treatment outcome to TNF inhibitors, were examined for association with treatment outcome.

Methods

RA patients
Clinical data of 325 patients enrolled in this study originated from a database of ApotheekZorg, which facilitates the Dutch distribution of adalimumab. In the Netherlands, adalimumab is prescribed according to the following protocol and is restrictive for reimbursement: 1) patients have used 2 DMARDs including MTX and 2) patients have a Disease Activity Score based on a 28-joint count (DAS28) of at least 3.2. Other inclusion criteria enclosed a diagnosis of RA according to the revised American College of Rheumatology criteria (ACR) (13); 18 years of age or older; an erythrocyte sedimentation rate (ESR) of at least 28 mm/hour; patient's global assessment of their general well-being measured on a 100 mm horizontal visual analogue scale (VAS), the left end representing as good as can be and the right end representing as worse as possible, of at least 20 mm.

The local ethics committee at our hospital (Leiden University Medical Center, The Netherlands) approved the study protocol. Patients gave written informed consent prior to inclusion.

SNP selection

The SNPs for analysis in this study were selected primarily according to a pathway selection method, as previously described (12). Briefly, a systematic approach was used including rational criteria for the selection of potential interesting SNPs within genes related to the mechanism of action of adalimumab. The criteria were based on the following characteristics of SNPs: heterozygosity frequency, validation status, ethnicity frequency, functionality based on alteration in protein, the extent of linkage disequilibrium and TAG SNPs. Included genes for SNP selection were genes encoding proteins involved in the mechanism of action of TNF inhibitors: neutralization and blockage (14;15); interaction with Fc receptor (cross-linkage) (16); initiation of reverse signaling, leading to blockage, increased apoptosis or growth arrest (17;18); reduction of inflammatory cytokine production and angiogenic factor expression (15;19-21); restoration of immune regulation (22); mediation of complement-dependent cytotoxicity (CDC) and antibody-dependent cytotoxicity (ADCC) (14;18); downregulation or discontinuation of bone and cartilage destruction (23;24). Ultimately, with this pathway approach 186 SNPs in 111 genes out of 51,793 SNPs in 124 genes were selected.
Additionally, a set of SNPs was selected (N=37), which included SNPs previously studied in relation with efficacy of TNF inhibitors and/or susceptibility to RA.

DNA collection and genotyping

After inclusion and with patients' written consent, DNA was obtained from patients by using DNA from single 2 ml salivary samples. Specifically, a sample of whole saliva was collected from each patient using a Oragene™ DNA self-collection kit (DNA Genotek Inc., Ottawa, Ontario, Canada). Hereafter, DNA samples were isolated according to a standard laboratory protocol for manual purification of DNA designed to optimize DNA yield (OrageneTM DNA Purification Protocol). DNA from 323 patients was suitable for genotyping. In 2 patients DNA was of low quality, which was defined as a DNA yield of lower than 10ug and/or nucleic acid purity (260nm/280nm ratio) lower than 1.6.
Genotyping was performed using a custom designed array with Veracode GoldenGate GT assays on the Illumina BeadXpress platform. This array interrogated 384 SNP loci within a single well of a standard microplate.
Before analysis for association, quality control procedures were performed: SNP genotyping plots for each assay were checked for degree of clustering (GenTrain score of <0.4) and samples with a call rate of less than 90% and assays with a call rate of less than 95% were removed. During this quality process, 22 SNPs and 8 patient samples were excluded for analysis.
Additionally, genotype frequencies of each assay were calculated and these distributions were tested for Hardy-Weinberg equilibrium (HWE) ($p<0.05$). If deviation from HWE was observed, genotype distribution in our population was compared with allele frequencies presented in the NCBI SNP database (http://www.ncbi.nlm.nih.gov/SNP). Accordingly, 5 SNPs were removed for analysis.

Clinical evaluation

For association analyses, primary and secondary endpoints were chosen based on two scales: change in 28 joint Disease Activity Score (DAS28) between baseline and at 14 weeks. As primary endpoints, achievement of good response and remission at 14 weeks according to EULAR criteria were chosen (25). EULAR good response was defined as a change of DAS28 ≥ 1.2 and DAS28 at 14 weeks ≤ 3.2. EULAR remission was defined as achieving DAS28 at 14 weeks ≤ 2.6. As secondary endpoint a relative change in DAS28 was selected ($DAS28_{baseline}$-$DAS28_{14weeks}$)/ $DAS28_{baseline}$).

Statistical Analysis

Initially, for the analysis of primary endpoints SNPs were explored for associations under allelic and genotypic model using chi-square tests. If a p-value of <0.1 was observed with either of these models, SNPs were fitted to the most appropriate model with Cochrane-Armitage test for trend- and logistic regression analyses for additive, recessive and dominant genetic model, respectively.

Secondly, if SNPs were significant SNPs ($p<0.05$) in the logistic regression analyses of either primary endpoints, the SNPs were candidates for analysis of the secondary endpoint. For this endpoint linear regression analyses for associations with relative change in DAS28 were applied.

Notably, with the analysis of SNPs previous associated with genetic susceptibility to RA and/or treatment outcome to TNF inhibitors, the reported genetic model (additive, recessive or dominant) was chosen. SNPs, which were reported under genotypic or allelic genetic model, were analyzed additionally according to methods described above.

All regression analyses were adjusted for age, gender, concomitant MTX therapy and DAS28 at baseline.

Since this study is explorative, no corrections for multiple testing were made. Therefore, p-values were presented without adjustments ($p <0.05$ was considered significant).

Statistical analyses were performed using SPSS 16.0 software (SPSS Inc., Chicago, IL, USA) and GPlink software (http://pngu.mgh.harvard.edu/purcell/plink/) (26).

Power calculation

With the appliance of a power calculation it was demonstrated that for our study population of 325 patients, minimal allele frequencies (MAF) ranging from 10% to 50%, an exposure (response of) of 45% and a chosen type 1 error probability $\alpha=0.05$, a power of >80% could be achieved to detect an odds ratio ranging from 1.4 (MAF of 50%) tot 2.0 (MAF of 10%). Notably, in more than 80% of the selected SNPs the MAF was higher than 30%

Results

Patient population

In total, 223 SNPs were genotyped in 323 patient samples, of which 196 (88%) SNPs were successfully genotyped in 315 (98%) patient samples after quality procedures and testing for HWE. Baseline demographic, disease characteristics and clinical response of the 325 initially included patients are presented in table 1. The average age of the cohort at the start of adalimumab therapy was 56 years with a mean disease activity (DAS28) at baseline of 5.9. Other patients received concomitant MTX with an average dose of 24.1 mg a week. In this cohort, 57 patients (18%) used adalimumab as monotherapy during evaluation period.
After 3 months of treatment with adalimumab, 174 (54%) and 103 (32%) of the patients responded according to the EULAR good response and EULAR remission criteria, respectively (table 1). No statistical differences in response rate between overall included patients and genotyped patients were observed (data not shown).

Characteristics	Value
Number of RA patients	325
Age -years (mean, sd)	56 (12)
Gender -female (%)	228 (71)
Concurrent MTX (%)	266 (82%)
MTX dose/week in mg (mean, sd)	24.1 (3.8)
Previous biological agent (%)	18 (6%)
DAS28 at baseline (mean, sd)	5.8 (0.98)
DAS28 at 3 months (mean, sd)	3.1 (1.1)
ΔDAS (mean, sd)	2.7 (1.0)
Relative change in DAS28 in % (mean)	46.1
Good response according to EULAR criteria (%)	174 (54)
Remission according to EULAR criteria (%)	103 (32)

Table 1. Study population characteristics[a,b]

a) Presented are numbers of patients (%) unless otherwise indicated.
b) Abbreviation(s): DAS28= 28 joint disaese activity score, MTX=methotrexate, EULAR= European League Against Rheumatism, sd= standard deviation

Primary endpoints

For EULAR good response, initial association analyses of 196 SNPs in 315 patients under allelic and genotypic model revealed 33 SNPs ($p < 0.1$) as candidates for further examination according to the best-fit genetic model. Additionally, multivariate regression analysis including covariates age, gender, concomitant MTX therapy and DAS28 at baseline, assessed for 19 SNPs significant associations with good response ($p<0.05$) (table 2). In this group, 2 polymorphisms in the *TANK* and *TNFAIP* genes were most significantly associated (for both SNPs $p=0.008$, table 2). When a multivariate analysis was performed with all 19 SNPs and covariates included, the SNPs *CD40LG* (rs1126535), *CSF1R* (rs10079250), *ICAM* (rs5498), *PECAM1* (rs4968622), *TANK* (rs1267067) and *TNFAIP3* (rs2230926) remained significant ($p<0.05$, table 2)

Gene	SNP (rs number)	MAF	Best-fit genetic model	P-value[b]	OR (95% C.I.)[b]
ADAM 17	rs1048610 (C>T)	0.47	Additive: CC > CT > TT	0,035	1,4 (1,0-2,0)
C3	rs344543 (G>C)	0.41	Additive: CC > CG > GG	0,044	1,4 (1,0-2,1)
CD40LG[c]	rs1126535 (T>C)	0.17	Recessive: T-allele vs. CC	0,013	5,1 (1,4-18)
CSF1R[c]	rs10079250 (A>G)	0.07	Dominant: AA vs. G-allele	0.037	2.1 (1.0-4.2)
FCRL3	rs7528684 (A>G)	0.42	Dominant: AA vs. G-allele	0,029	1,7 (1,1-3,0)
ICAM1[c]	rs5498 (A>G)	0.42	Dominant: AA vs. G-allele	0,048	1,6 (1,0-2,7)
IL1A	rs1304037 (A>G)	0.33	Recessive: A-allele vs. GG	0,012	3,2 (1,3-7,9)
IL1RAP	rs13321840 (T>G)	0.29	Additive: TT>GT>GG	0,011	1,6 (1,1-2,4)
IL4R	rs1049631 (A>G)	0.47	Recessive: GG vs. A-allele	0,022	2,0 (1,1-3,6)
KDR	rs6828477 (T>C)	0.41	Dominant: TT vs. C-allele	0,041	1,4 (1,0-2,1)
PADI4	rs2240340 (G>A)	0.42	Dominant: GG vs. A-allele	0,013	1,9 (1,1-3,2)
PECAM1[c]	rs4968622 (G>A)	0.45	Dominant: A-allele vs. GG	0,020	1,9 (1,1-3,2)
TANK[c]	rs1267067 (T>C)	0.32	Additive: CC>CT>TT	0,008	1,7 (1,1-2,5)
TGFB1	rs1800469 (C>T)	0.28	Recessive: C-allele vs. TT	0,027	3,5 (1,1-10,5)
TGFB1	rs2241715 (G>T)	0.27	Recessive: G-allele vs. TT	0,028	3,4 (1,1-10)
TNFAIP3[c]	rs2230926 (T>G)	0.04	Additive: TT>GT>GG	0,008	4,0 (1,4-11)
TNFRSF11B	rs11573885 (G>A)	0.43	Additive: AA>AG>GG	0,036	1,5 (1,0-2,1)
TNFRSF11B	rs1485286 (T>C)	0.29	Additive: CC>CT>TT	0,036	1,5 (1,0-2,2)
VEGFA	rs25648 (C>T)	0.16	Additive: TT>CT>CC	0,021	1,8 (1,1-3,0)

Table 2. Association between SNPs and adalimumab efficacy (EULAR good response at 14 weeks) according to the best-fit genetic model (p<0.05)[a,b,c]

a) Abbreviation(s): EULAR= European League Against Rheumatism, MAF= minimal allele frequency, OR= odds ratio, 95% C.I.= 95 % confidence interval, SNP=single nucleotide polymorphism. Abbreviations and accessory full names of formal genes can be relocated in the NCBI gene database *(http://www.ncbi.nlm.nih.gov)*.
b) P-values and odds ratios with 95% confidence intervals are calculated by logistic regression analysis, adjusted for baseline DAS28, gender, age and concomitant MTX treatment according to best-fit genetic model.
c) When a multivariate analysis was performed with all 19 SNPs and covariates included, these SNPs remained significant (p<0.05)

Regarding the analysis of association with EULAR remission, 27 SNPs of the included 196 SNPs were further analysed (p<0.1) under additive, recessive and dominant genetic model. Within this set of potential SNPs, 11 SNPs were significantly associated with remission (p<0.05, table 3). Specifically, associations were observed with *CD69* (rs10844706, p= 0.035), *FLT1* (rs748253, p=0.031), *PTPN22* (rs2476601, p= 0.031), Region Chr1p34 (rs17534243, p= 0.010), *TANK* (rs1267067, p=0.006) and *VEGFA* (rs25648, p= 0.045) according to an additive genetic model; with *KDR* (rs6828477, p=0.016), *PECAM1* (rs12953, p=0.042), *RSBN1* (rs6679677, p=0.030) and *VWF* (rs1063856, p=0.022) according to a dominant genetic model and with *CD40LG* (rs1126535, p=0.048) according to a recessive genetic model (table 3). When a multivariate analysis was performed with all 11 SNPs and covariates included, the SNPs *KDR* (rs6828477), *PECAM1* (rs12953), Region Chr 1p34 (rs17534243), *TANK* (rs1267067) and *VWF* (rs1063856) remained significant (p<0.05, table 3).

Gene	SNP (rs number)	MAF	Best-fit genetic model	P-value[b]	OR (95% C.I.)[b]
CD69	rs10844706 (C>A)	0.36	Additive: AA>AC>CC	0.035	1.5 (1.0-2.3)
CD40LG	rs1126535 (T>C)	0.17	Recessive: T-allele vs. CC	0.048	1.9 (1.0-3.6)
FLT1	rs748253 (G>T)	0.40	Additive: TT>GT>GG	0.031	2.2 (1.1-4.4)
KDR[c]	rs6828477 (T>C)	0.41	Dominant: TT vs. C-allele	0.016	2.0 (1.1-3.5)
PECAM1[c]	rs12953 (A>G)	0.48	Dominant: AA vs. G-allele	0.042	1.8 (1.0-3.3)
RSBN1	rs6679677 (C>A)	0.17	Dominant: CC vs. A-allele	0.030	2.0 (1.1-3.7)
PTPN22	rs2476601 (G>A)	0.17	Additive: GG>AG>AA	0.038	1.8 (1.0-3.1)
Region Chr1p34[c]	rs17534243 (A>G)	0.23	Additive: GG>AG>AA	0.016	1.7 (1.1-2.7)
TANK[c]	rs1267067 (T>C)	0.32	Additive: CC>CT>TT	0.006	1.8 (1.2-2.7)
VEGFA	rs25648 (C>T)	0.16	Additive: TT>CT>CC	0.045	1.7 (1.0-3.0)
VWF[c]	rs1063856 (A>G)	0.34	Dominant: AA vs. G-allele	0.022	1.9 (1.1-3.3)

Table 3. Association between SNPs and adalimumab efficacy (EULAR remission at 14 weeks) according to the best-fit genetic model (p<0.05)[a,b,c]

a) Abbreviation(s): EULAR= European League Against Rheumatism, OR= odds ratio, 95% C.I.= 95 % confidence interval, SNP=single nucleotide polymorphism. Abbreviations and accessory full names of formal genes can be relocated in the NCBI gene database *(http://www.ncbi.nlm.nih.gov)*.
b) P-values and odds ratios with 95% confidence intervals are calculated by logistic regression analysis, adjusted for baseline DAS28, gender, age and concomitant MTX treatment according to best-fit genetic model.
c) When a multivariate analysis was performed with all 11 SNPs and covariates included, these SNPs remained significant (p<0.05)

Secondary endpoints

In total, 30 SNPs were examined for association with a relative change in DAS28 according to the SNPs' best-fit model. Linear regression analyses revealed 8 significant associations (p<0.05). Specifically, associations were demonstrated with *ADAM17* (rs1048610, p= 0.002), *TNFRSF11B* (rs1485286, p=0.019), *TANK* (rs1267067, p= 0.010) and *VEGFA* (rs25648, p= 0.025) according to an additive genetic model; with *FCRL3* (rs7528684, p= 0.004), *KDR* (rs6828477, p= 0.003), *PECAM* (rs12953, p= 0.026) according to a dominant genetic model and with *CD40LG* (rs1126535, p= 0.016) according to a recessive genetic model (data not displayed in table).
Notably, four SNPs were significantly associated with the analyses according to all three endpoints: *CD40LG* (rs1126535), *KDR* (rs6828477), *TANK* (rs1267067) and *VEGFA* (rs25648) (table 4).

	MAF	EULAR Good response		EULAR Remission		Relative decrease of DAS28 in %	
		P-value	*OR (95% C.I.)*	*P-value*	*OR (95% C.I.)*	*Response differences in %*	*P-value*
***CD40LG* (rs1126535)** Recessive genetic model: T-allele carriers vs. CC	0.17	0.013	5,1 (1,4-18)	0.048	1.9 (1.0-3.6)	46.8 vs. 36.9	0.016
***KDR* (rs6828477)** Dominant genetic model: TT vs. C-allele carriers	0.41	0,041	1,4 (1,0-2,1)	0.016	2.0 (1.1-3.5)	49.9 vs. 44.2	0.003
***TANK* (rs1267067)** Additive genetic model: CC>CT>TT	0.32	0,008	1,7 (1,1-2,5)	0.006	1.8 (1.2-2.7)	44.1>52.3>54.8	0.010
***VEGFA* (rs25648)** Additive genetic model: TT>CT>CC	0.34	0,021	1,8 (1,1-3,0)	0.045	1.7 (1.0-3.0)	44.9>54.0>57.9	0.025

Table 4. Association of SNPs in *CD40LG*, *TANK, VEGFA* and *KDR* according to EULAR criteria and relative change in DAS28[a,b]

a) Abbreviation(s): EULAR= European League Against Rheumatism, OR= odds ratio, 95% C.I.= 95 % confidence interval, SNP=single nucleotide polymorphism. B= regression coefficient, S.E.= standard error, KDR= kinase insert domain receptor, MAF= minimal allele frequency, TANK= TRAF family member-associated NFKB activator, VEGF= vascular endothelial growth factor, DAS28= 28-joint count disease activity score.
b) P-values, odds ratios with 95% confidence intervals and regression coefficients with standard errors are calculated by logistic regression analysis and linear regression analysis, respectively. In these analyses adjustments for baseline DAS28, gender, age and concomitant MTX treatment according to best-fit genetic model were made.

Discussion

In this explorative study, our aim was to determine whether SNPs in genes related to the mechanism of action of adalimumab were associated with efficacy of this biological DMARD. Furthermore, SNPs from previous research on pharmacogenetics of TNF inhibitors in RA and susceptibility to RA were included for analysis. It was demonstrated that 19 SNPs, 11 SNPs and 8 SNPs were significantly associated with EULAR good response, EULAR remission and relative change in DAS28, respectively. Four SNPs, rs1126535 in *CD40LG*, rs6828477 in *KDR*, rs1267067 in *TANK* and rs25648 in *VEGFA* showed consistent associations and, therefore, they appear to be the most predictive for adalimumab efficacy.

The pathway pharmacogenetic method used in this study has an advantage over the classical usage of the candidate gene approach and the whole genome approach. The main feature of this method is that a set of SNPs is selected based on a description of pathways regarding the mechanism of action of the drug under study (12). With the candidate gene method, SNPs that are responsible for the rate limiting or extending step in the mechanism of action of adalimumab are easily missed. Therefore, it seems likely that for most drugs and complex diseases, like RA, pharmacogenetics has more potential if information on multiple genes is used. Numerous proteins are involved in the mechanism of action and, therefore, genetic variability in each gene may contribute to the overall variability in drug response.

The four most potential SNPs, *CD40LG* (rs1126535), *KDR* (rs6828477), *TANK* (rs1267067) and *VEGFA* (rs25648) are rs numbers close to genes encoding proteins involved in the mechanism of action of adalimumab. CD40LG is a member of the TNF superfamily, which is primarily expressed on the surface of activated T-cells and stimulates B-cell proliferation and secretion of proinflammatory cytokines and chemokines after CD40-CD40LG ligation (27). CD40LG is an extracellular target for TNF inhibitors. This was also demonstrated in a study of Danese et al (28), which measured reduced levels of CD40LG with infliximab. Correspondingly, adalimumab may limit the inflammatory process involved in RA by inhibiting CD40LG. Likewise, the observed interindividual differences in response in our study may be the result of the synonymous SNP rs1126535 in the gene encoding the CD40LG protein. Concerning our results, patients genotyped for CD40LG (rs1126535) CC may have less binding capacity to TNF inhibitors compared with patients genotyped carrying a *CD40LG* (rs1126535) T-allele (table 4).

Vascular endothelial growth factor (*VEGF*) is an important protein involved in angiogenesis. Since it is demonstrated that expression of *VEGF* is reduced in RA patients treated with TNF inhibitors, it is thought that these biologicals may interfere with the angiogenesis seen in the inflammatory process of RA (15). Regarding our selected SNP *VEGFA*-rs25648, patients genotyped for a homozygous mutant genotype (rs25648-TT) achieved a better response than patients genotyped for carrying the wildtype C-allele (rs25648-CT, rs25648-CC). Hypothetically, the mutant genotypes may assist TNF inhibitors in reduction of angionesis in RA by facilitating decreased expression of VEGFA enzyme.

Also, VEGF acts by binding with to tyrosine kinase receptors (VEGFR), of which KDR (kinase insert domain receptor) is the primary receptor for mediation of VEGF's action. In the study of Cañete et al it was observed that reduction of synovial angiogenesis after therapy with TNF inhibitor therapy in psoriatic arthritis patients could be related to modulation of molecular factors, like KDR, involved in this process (29). Hypothetically, regarding our results for rs6828477, indicate that carrying the C-allele may have a decreased modulation of KDR by adalimumab leading to a lower response compared with patients with a TT genotype.

In addition, TANK, a protein coding for TRAF family member-associated NFKB activator, is part of the TRAF family which mediates signals from cytokines signals through cell surface receptors and, hereby, activating downstream intracellular signaling cascades. TANK binds to TRAF1, TRAF2 and TRAF3. It is seen that the mechanism of action of TNF inhibitors includes the initiation of reverse intracellular signaling cascade by binding of antibodies to transmembrane TNF. This may lead to a decreased production of proinflammatory cytokines (like TNFα), increased production of anti-inflammatory cytokines (like IL10) and induction of apoptosis in cells (14;15;30). In our results, patients with a homozygous mutant genotype (rs1267067-CC) were more likely to achieve clinical response than patients carrying a wildtype allele (rs1267067-TT or rs1267067-CT). Hypothetically, this polymorphism may support the reverse signaling by less binding of TRAF1-TRAF3 to TANK. Generally, along with replication of these explored SNPs in other patient cohorts and determination whether these SNPs provide the strongest association compared with other adjacent variants in the gene, functional studies remain to be performed (31). In this way, beneficial- and/or risk genotypes involved in efficacy of TNF inhibitors could be biologically identified.

Concerning susceptibility to RA, GWA studies have provided new insights in the genetic risk profile of an individual RA patient. These genetic variants have strong evidence to be associated with RA and include SNPs in the *TRAF1-C5*, *OLIG3-AIP3* and *STAT4* gene regions. Consequently, genetic findings led to improved knowledge regarding the pathogenesis of this immune-mediated disease. Likewise, these findings could have implications on pharmacogenetics in RA. As is seen in other medical research areas, it seems plausible that a pharmacogenetic response in RA depends on the interaction of genes involved in anti-rheumatic drug metabolism and genes associated with RA's pathogenesis (32;33). However, several studies have been performed on the predictive value of these variants for response to therapy with TNF inhibitors, but with inconclusive results to answer the question whether in general genes contributing to disease susceptibility may play a role in determining response to treatment. In our analyses, associations were detected with *TNFAIP* (rs2230926), *PADI4* (rs2240340), *FCRL3* (rs7528684), *RSBN1* (rs6679677), Region Chr. 1p34 (rs17534243) (tables 2 and 3). Still, these results were not as consistent as the results for rs1126535 (*CD40LG*), rs6828477 (*KDR*), rs1267067 (*TANK*) and rs25648 (*VEGFA*) based on all three endpoints.

In our study, no adjustment for multiple testing was performed. Assessing a large number of SNPs for association creates potential false positive results and additionally the need for adjustment for multiple testing. Generally, the need for multiple testing arises from the assumption that the incidence of false positives is proportional to the number of test performed and level of significance. The most common method for dealing with multiple testing, the Bonferroni correction, involves adjusting the significance level of each test by the total number of performed tests (34). However this method has a conservative character, since interaction and cooperation between causative genes are not recognized (35;36). Consequently, studies may be willing to risk higher frequencies of false positives, instead of finding no associations at all due to false negative associations. Ideally, replication in a second comparable cohort of patients would be optimal, but not always feasible. Therefore, we have decided to present the p-values of this explorative study without adjustments to make the results accessible for clear interpretation.

Still, we would like to underline that we have performed multiple independent tests (e.g. variable endpoints) and that we recognize that the consideration of a cutoff value of $p=0.05$ for level of significance in this study is inaccurate. Therefore our results need to be marked as suggestive for association with adalimumab efficacy.

Generally, future research should be done to confirm our significant results. Particularly, replication of our results in patients treated with different TNF inhibitors remains a challenge. However, recently in a report from Bowes et al (7), stratified analysis for infliximab and etanercept was per-

formed. No significant differences were observed, suggesting that response was not subject to drug-specificity. Moreover, their results confirm the general applicability of our pathway pharmacogenetic approach, since this approach is based on the mechanism action of the class of TNF inhibitors.
In conclusion, primary analyses in this study have revealed new SNPs, rs1126535 in *CD40LG*, rs6828477 in *KDR*, rs1267067 in *TANK* and rs25648 in *VEGFA*, which may be involved in the efficacy of adalimumab. In this way, the results from this explorative study provide new insights regarding the potential of pharmacogenetics of adalimumab in RA.

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