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Investigation of the influence of *CYP1A2* and *CYP2C19* genetic polymorphism on A771726 pharmacokinetics in leflunomide treated patients with rheumatoid arthritis

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Running title: *CYP1A2* and *CYP2C19* polymorphism and A77 1726 pharmacokinetics

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List of nonstandard abbreviations: A77 1726, leflunomide metabolite; ADRs, adverse drug reactions; ALT, alanine aminotransferase; anti-CCP, anti-citrullinated peptide antibodies; BSA, body surface area; CL/F, oral clearance; CRP, C-reactive protein; CI, confidence intervals; C_{ss}, average steady-state concentration; CYPs, cytochromes P450; DAS28, disease activity score of 28 joint count; DMARDs, disease-modifying antirheumatic drugs; ESR, erythrocyte sedimentation rate; GFR, glomerular filtration rate; k_a, absorption rate constant; k_e, elimination rate constant; MTX, methotrexate; NSAIDs, non-steroidal anti-inflammatory drugs; RA, rheumatoid arthritis; RF, rheumatoid factor; RFLP, restriction fragment length polymorphism; SNPs, single

nucleotide polymorphisms; V/F, volume of distribution; VAS, visual analogue scale; WT, body weight

Abstract

Leflunomide is a disease-modifying antirheumatic drug used for the treatment of rheumatoid arthritis (RA). Cytochromes P450, mainly CYP1A2 and CYP2C19 might be involved in the transformation of leflunomide to leflunomide metabolite (A77 1726). The aim of this study was to investigate if genetic polymorphisms in *CYP1A2* and *CYP2C19* influence leflunomide pharmacokinetics, treatment response and the occurrence of adverse drug reactions (ADRs). The study included 67 RA patients and 4 patients with polyarthritis resembling RA and psoriasis treated with leflunomide. A77 1726 steady-state plasma concentrations were determined by validated HPLC with UV detection. A population pharmacokinetic model was developed to estimate the oral clearance (CL/F) and volume of distribution (V/F). A genotyping approach was used to determine C-163A, C-729T and T-739G in the *CYP1A2* gene as well as SNPs that characterize *CYP2C19 *2*, *3, *4* and *17* alleles. A large inter-individual variability in trough A77 1726 steady-state plasma concentrations was observed (from 1.9 to 156.9 mg/L). A77 1726 CL/F was 71% higher in carriers of *CYP2C19*2* allele compared to non-carriers. A77 1726 average steady-state plasma concentration was associated with the treatment response. Patients with greater decrease in C-reactive protein (CRP) had higher average steady-state plasma A77 1726 concentrations, 49.7±39.0 mg/L in patients with ΔCRP of more than 8.5 mg/L compared to 24.8±13.7 mg/L in patients with ΔCRP of less or equal to 8.5 mg/L ($p = 0.015$). No association of A77 1726 steady-state plasma concentrations with the occurrence of ADRs was observed. Our results suggest that genetic variability in leflunomide-metabolizing enzymes influence leflunomide metabolite concentrations that are associated with the treatment response, but not with leflunomide-induced toxicity.

Introduction

Leflunomide [5-methyl-N-[4-(trifluoromethyl)phenyl]-4-isoxazolecarboxamide] is a disease-modifying antirheumatic drug of the isoxazole class used for the treatment of rheumatoid arthritis (RA). *In vivo* it is converted to its active metabolite A77 1726 [2-cyano-3-hydroxy-N-[4-(trifluoromethyl)phenyl]-2-butenamide] (Figure 1) that inhibits dihydroorotate dehydrogenase (DHODH), a rate-limiting enzyme in *de novo* synthesis of pyrimidine (Mladenovic et al., 1995). Clinical trials, reviewed by Kunkel and Cannon (Kunkel and Cannon, 2006), have shown that leflunomide efficacy is comparable with methotrexate (MTX) and sulfasalazine. However, the rate of withdrawal from leflunomide therapy was found to range between 40% and 70% in the first year due to the development of adverse drug reactions (ADRs) and lack of efficacy.

Leflunomide acts as a prodrug. Following oral administration it is rapidly and almost completely absorbed and predominantly pre-systemically metabolized to A77 1726. Plasma levels of leflunomide are observed only occasionally at very low concentrations. Peak concentrations of the active metabolite occur 6 to 12 hours after dosing. The metabolite is 99% bound to plasma albumin resulting in relatively low apparent volume of distribution (V/F) ranging between 6 and 30.8 L, with an average of 12.7 L (Rozman, 2002). The active metabolite of leflunomide is eliminated by further metabolism to glucuronides of methyl-hydroxy A77 1726 and trifluoromethylanine-N-oxanillic acid, as well as direct excretion in the bile and urine. The urinary and billiary routes of elimination are of similar importance. The clearance (CL/F) of A77 1726 is approximately 0.020 L/h and is dose independent, resulting in an elimination half-life of 14 to 18 days. Entero-hepatic circulation notably contributes to long elimination half-life

as some studies indicate that activated charcoal and cholestyramine reduce A77 1726 plasma half-life down to approximately one day. Large inter-individual variability in leflunomide pharmacokinetics was observed, that can be partially explained by variation in patient age, gender, body size, liver function and smoking status (Chan et al., 2005; Shi et al., 2005). So far, influence of genetic variability in enzymes involved in leflunomide metabolic pathway on leflunomide pharmacokinetics has not been investigated.

A study on human liver microsomes suggested that cytochromes P450 (CYPs) may be involved in leflunomide activation and CYP1A2, CYP2C19 and CYP3A4 appear to be the major enzymes responsible for leflunomide metabolism (Kalgutkar et al., 2003). Single nucleotide polymorphisms (SNPs) in genes coding for *CYP1A2*, *CYP2C19* and *CYP3A4* have been described that may modify metabolic activity. Although wide interindividual variability in CYP1A2 activity has not been unequivocally linked to any polymorphic site in the *CYP1A2* gene, among more than 20 SNPs identified, C-163A (rs762551), C-729T (rs12720461) and T-739G (rs2069526) have been suggested to affect *CYP1A2* expression or enzymatic activity (Sachse et al., 1999; Aklillu et al., 2003). SNPs in *CYP2C19* gene that characterize **2, *3, *4* alleles result in an abolished enzyme activity, while SNP that characterizes **17* allele was associated with increased enzyme activity (De Morais et al., 1994a; de Morais et al., 1994b; Ferguson et al., 1998; Sim et al., 2006). In the *CYP3A4* gene however, no major functionally variant allele has been found in Caucasians at an allele frequency higher than 0.1% and only limited genotype-phenotype association has been observed (He et al., 2005).

We have previously shown that SNPs in *CYP1A2* but not in *CYP2C19* are associated with the occurrence of leflunomide-induced ADRs (Bohanec Grabar et al., 2008). The aim of

the present study was to develop a population pharmacokinetic model of A77 1726 in the cohort of patients with RA treated with leflunomide to evaluate the influence of SNPs in *CYP1A2* and *CYP2C19* and other demographic and biochemical parameters on A77 1726 pharmacokinetics and to analyze relationship between A77 1726 steady-state plasma concentrations with disease activity and occurrence of ADRs.

Methods

Patients and study design

The study recruited 71 consecutive unrelated adult Central European Caucasian patients, of whom 67 were diagnosed with RA and 4 patients with polyarthritis resembling RA. The patients were treated at the Department of Rheumatology, University Medical Centre Ljubljana, Slovenia and were enrolled from September 2006 to September 2007. The study was approved by the Slovenian Ethics Committee for Research in Medicine and was carried out according to the Helsinki Declaration. All patients gave their written informed consent prior to enrolment.

All patients were on maintenance therapy with leflunomide 10 or 20 mg/day. In the majority of patients leflunomide therapy was initiated with a loading dose of 60 or 100 mg/day for 3 days and then continued with a recommended dose of 20 mg/day. In some patients the leflunomide dose was reduced to 10 mg/day, since dosing at 20 mg/day was not tolerated clinically. A detailed history of leflunomide dosing, including the duration of leflunomide therapy and all other concomitant medications were collected from patients' charts. Compliance was assessed by an interview with the attending physician.

The study was cross sectional by design and A77 1726 pharmacokinetics, disease activity and other clinical measurements were assessed at one time in each patient. ADRs were recorded retrospectively from patients' files throughout the course of the leflunomide treatment. The occurrence of ADRs was re-evaluated one year after the beginning of the study.

Rheumatoid factor (RF) was determined by the Waaler- Rose and Latex agglutination method and the presence of anti-citrullinated peptide antibodies (anti-CCP) was assessed with the ELISA method using Immunoscan RA anti-CCP test kit (Euro-Diagnostica, Malmö, Sweden). Alanine aminotransferase (ALT), S-creatinine concentration, erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) were measured as part of the standard patient care. Glomerular filtration rate (GFR) was calculated by the modified diet in renal disease (MDRD) study equation (Levey et al., 2006). Treatment response was evaluated by the disease activity score of 28 joint count (DAS28), calculated for each patient based on the number of tender and swollen joints, ESR and patients' global assessment of general health expressed on visual analogue scale (VAS). Cigarette smoking status was determined by patient interview. Data on ESR and CRP levels at the initiation of leflunomide treatment, duration of disease, patient age, body height and weight; and erosive changes of hands and feet observed on X-rays were collected from individual patients' charts. Body surface area (BSA) was estimated by Mosteller formula (Mosteller, 1987).

Three blood samples were taken from each patient, one pre-dose, and two at approximately 3 and 6 hours after the leflunomide dose. The blood samples were centrifuged at 2000 g for 10 min for plasma separation. Plasma samples were stored at -

20°C and were used for the pharmacokinetic analyses, while cellular fractions were stored for a short term at -20°C until DNA isolation.

Genotyping

Genomic DNA was isolated from peripheral blood leukocytes using a Qiagen FlexiGene kit (Qiagen GmbH, Germany). A genotyping approach using restriction fragment length polymorphism (RFLP) was used to analyze *CYP1A2* and *CYP2C19* polymorphisms. To determine the *CYP1A2* C-163A polymorphism the region containing polymorphic site was amplified and PCR product was digested with *Apa*I as described previously (Sachse et al., 1999). The region encompassing *CYP1A2* polymorphic sites -729 and -739 was amplified simultaneously and the PCR product was digested with restriction enzymes *Ava*II and *Nci*I (New England Biolabs) to determine C-729T and T-739G polymorphisms, respectively (Aklillu et al., 2003). In *CYP2C19* the following SNPs were analysed; G681A (rs4244285), G636A (rs4986893), A1G (rs28399504) and C-806T (rs12248560) to characterize **2*, **3*, **4* and **17* alleles, respectively. To determine G681A, G636A and A1G SNPs the regions encompassing polymorphic sites were amplified in separate PCR reactions and PCR products were further digested with *Sma*I, *Bam*HI and *Pst*I (New England Biolabs), respectively as previously described (De Morais et al., 1994a; de Morais et al., 1994b; Ferguson et al., 1998). To determine C-806T SNP real-time PCR was performed using the primer and probe sets as previously reported (Bohanec Grabar et al., 2008).

A77 1726 analysis

Analytical standard of A77 1726 was synthesized from 5-methyl-4-isoxazolecarbonyl chloride and 4-(trifluoromethyl)aniline (Across Organics, Morris Plains NJ). In brief 4-(trifluoromethyl)aniline was dissolved in toluene, heated to 60°C, then equimolar amount of 5-methyl-4-isoxazolecarbonyl chloride was added and the reaction was left for 2 hours to form leflunomide. In the subsequent reaction leflunomide was converted to A77 1726 in 1 M NaOH in methanol over 24 h. A77 1726 was filtered, washed and recristalized from toluene. Identity of A77 1726 was confirmed by element analysis, mass spectometry and ^{1}H-NMR. Purity of analytical standard assessed by HPLC was at least 98.5%.

Agilent HP 1100 (Waldbronn, Germany) HPLC system equipped with vacuum degasser, binary pumps, auto sampler and diode array detector was used to measure A77 1726 in plasma. To 100 μL plasma samples 40 μL of trans-4-(trifluoromethyl)cinnamic acid (Sigma-Aldrich, Steinheim, Germany) used as internal standard solution in carbonate buffer (150 mg/L) were added and vortexed for 15 seconds. For precipitation of plasma proteins 200 μL of acetonitrile was added, vortexed for 30 seconds and left on ice for 20 minutes. To precipitated plasma samples 300 μL of phosphate buffer pH 2 was added and centrifuged at 16000 g for 10 minutes. 25 μL of the clear supernatant solution was injected into HPLC system. Chromatographic separation was achieved on Chromolith Performance column (RP-C18, 100 mm x 3 mm) attached to Chromolith Performance precolumn (RP-C18, 5 mm x 4.6 mm) (Merck, Darmstadt, Germany). Mobile phase consisted of acetonitrile and phosphate buffer (3.4 g orthophosphoric acid (85%), 2.8 g potassium dihydrogen phosphate, pH 2) (35/65, v/v) and flow rate was set at 1.7 ml/min. Analysis run time was 4.5 min. Analytes of interest were detected at 270 nm (I.S.) and 292 nm (A77 1726). Calibration lines were calculated from A77 1726 to I.S. area ratios.

Method was linear in the range between 1 and 200 mg/L. Limit of quantification was 1 mg/L. Intra- and inter-day variability of the method at nominal concentrations of 2, 20 and 200 mg/L was less than 8.9% (RSD) and mean recovery (n = 3) was 101.8 ± 2.4%. Stability of A77 1726 in plasma samples was at least 6 months (<5% difference), if stored at -20°C.

Pharmacokinetic analysis

Pharmacokinetic analysis was performed by a population modelling approach by using NONMEM software (version 6, level 2, GloboMax LLC, Ellicott City, MD, USA) and Perl speaks NONMEM (version 2.3, http://psn.sourceforge.net) (Lindbom et al., 2005). Fortran subroutines were compiled with the Intel Visual Fortran compiler, version 10.1 (Intel, Santa Clara, CA, USA). A77 1726 plasma concentration data were fitted by a one-compartment model with first-order absorption and first-order elimination, which was specified by NONMEM subroutines ADVAN2 and TRANS2. The first-order conditional method with interaction was used for estimation of apparent CL/F and V/F. Due to insufficient data in the absorption phase, absorption rate could not be estimated. Therefore, the absorption rate constant (k_a) was fixed at 1 h^{-1} as estimated using the following relationship: $t_{max} = \ln(k_a/k_e)/(k_a-k_e)$, based on a literature value of plasma elimination half-life of approximately 2 weeks, corresponding to elimination rate constant (k_e) of 0.0021 h^{-1}, and t_{max} of 6-12 h. To assess the potential effect of misspecification of k_a on other population pharmacokinetic parameters in the model, sensitivity analysis was conducted with values for k_a ranging from 0.2-5 h^{-1}. The base model without covariates was used initially to describe A77 1726 plasma concentration-

time data and to obtain estimates for A77 1726 CL/F and V/F. An exponential error model was used to describe the inter-individual variability in CL/F ($\omega^2_{CL/F}$) and V/F ($\omega^2_{V/F}$), while additive, proportional and combination error models were evaluated to describe residual variability of A77 1726 concentration (σ^2), which includes intra-individual variability, inter-occasional variability, and errors in dosing, sample collection, and assay.

During base model building, case deletion diagnostics were used to detect the outliers in the data (Lindbom et al., 2005). For all subjects in a study Cook's score and covariance ratio were computed. Cook's score is a measure of how much the model's parameters change when the particular subject's data are removed from the dataset, while covariance ratio measures the decrease in parameters' precision. A high Cook's score with a low covariance ratio indicates an outlier.

In the univariate analysis, the effects of body weight (WT), BSA, gender, age, GFR, ALT, duration of disease and duration of leflunomide treatment, smoking status, co-treatment with non-steroidal anti-inflammatory drugs (NSAIDs), methotrexate (MTX) corticosteroids and proton pump inhibitors, and genetic polymorphism of *CYP1A2* and *CYP2C19* were investigated. Continuous covariates were included in the base model using a power relationship centred to the value that was close to the mean in the studied population of patients. Categorical covariates (gender, co-treatment and smoking status) were assigned values of 0 and 1 and were modelled to estimate proportional change in the typical value of the pharmacokinetic parameters. The associations of various genotypes were tested in a similar manner. Effect of each allele combination was evaluated as proportional change in model parameters against homozygous wild type allele.

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The model adequacy was evaluated by standard diagnostic plots. Additional criteria were convergence of minimization, number of significant digits more than 3, successful covariance step and gradients in the final iteration in the range between 10^{-3} and 10^{2}. Hierarchical models were compared by the likelihood ratio test. Criterion for selection of a model was a change in minimum value of objective function (ΔOFV), which is approximately χ^2 distributed, with degrees of freedom that is equal to the difference in the number of parameters between the two models (at least 3.84 per one additional parameter, corresponding to $p < 0.05$).

Significant covariates according to the likelihood ratio test were rank-ordered and introduced into the full model. The final model was determined by testing each covariate against the full model using a likelihood ratio test to see if it should remain in the model. Additional criterion for the retention of a covariate in the model was reduction in the unexplained inter-individual variability.

The final model was evaluated by the visual predictive check and the bootstrap method. Bootstrap sampling method with replacement using 2000 replications was applied to determine the 2.5^{th} and 97.5^{th} percentile for each of the population parameters and were reported as 95% confidence intervals (CI).

Statistical analysis

To explore the relationship between A77 1726 pharmacokinetics and its clinical effects, Bayesian estimates of A77 1726 CL/F were used to calculate average steady-state concentration (C_{ss}) in individual patient. Patients were divided at the median values of the clinical assessments into low and high group and differences in A77 1726 C_{ss} between

the groups were tested by Mann-Whitney U test. Significance level was set at $p < 0.05$. Statistical analysis was performed using SPSS 15.0 for Windows (SPSS, Inc., Chicago, IL).

Results

The study enrolled 71 patients and their demographic and clinical data are presented in Table 1. In the majority of patients the maintenance leflunomide dose was 20 mg/day. In 6 patients the dose was permanently or intermittently lowered to 10 mg/day due to toxicity and/or concomitant treatment with other DMARDs. During the first year of the study 5 patients developed severe ADRs and were discontinued from leflunomide treatment, while 5 patients developed mild to moderate ADRs with 9 clinical manifestations, the majority of them being hypertension (3 cases) and were continued on leflunomide treatment.

We analyzed three SNPs in the *CYP1A2* gene; C-163A, C-729T and T-739G and four in *CYP2C19* gene specific for **2, *3, *4* and **17* allele. No individual with *CYP2C19*3* or *CYP2C19*4* allele was identified. The distribution of *CYP1A2* and *CYP2C19* genotypes in RA patients is shown in Table 2. The observed frequencies of the studied polymorphisms did not deviate from the Hardy-Weinberg equilibrium. Further on, *CYP1A2* and *CYP2C19* haplotypes were constructed. Under the assumption of linkage disequilibrium in *CYP1A2* three most common haplotypes were found with the following expected frequencies: **1A* 32.6%, **1F* 64.4% and **1J* 2.2%. Expected frequencies of *CYP2C19* alleles were **1* 57.7%, **17* 33.8% and **2*8.5%. The observed frequencies of *CYP1A2*1F, CYP1A2*1J* and *CYP2C19*2* alleles in our patient group were similar to

the frequencies reported for the healthy Caucasian and Slovenian populations, respectively (Herman et al., 2003; Skarke et al., 2005). The observed frequencies of *CYP2C19*17* allele were higher in RA patients than the ones reported in the International HapMap project for healthy Caucasian population (33.8% vs. 21.7%), however not significantly.

Due to very long elimination half-life, A77 1726 concentration profiles were very flat with little variation within an individual patient. The difference between maximum and minimum A77 1726 plasma concentration in an individual patient ranged between 1.2 and 18.2%. However, there was significant inter-individual variability, and A77 1726 trough plasma concentrations ranged between 1.9 and 156.9 mg/L, with a mean of 33.7 ± 28.9 mg/L. Six patients were suspected for poor drug compliance, but were nevertheless included in the initial pharmacokinetic analysis. A77 1726 plasma concentration profiles were best described with a one-compartment model with absorption rate constant fixed at 1 h^{-1}, exponential model for inter-individual variability in CL/F and a combination error model, comprising an additive and proportional component for residual, intra-individual variability. Inter-individual variability in V/F could not be estimated due to parameter shrinkage. Case-deletion diagnostics revealed that 2 of the 6 subjects previously suspected for poor drug compliance, are outliers with a covariance ratio of less than 0.4 and a Cook's score of more than 1. Consequently, these two subjects' data were excluded from the further analysis. With the base model developed on the remaining data from the 69 patients, CL/F was estimated at 0.0302 L/h and an inter-individual variability of 78%, while V/F was estimated at 8.55 L. Residual variability in A77 1726 concentration was 7.48% (proportional) and 0.250 mg/L (additive).

Observation of the plots of posterior Bayesian estimates of CL/F versus various covariates indicated a trend of higher CL/F in patients with higher GFR estimated by the modified MDRD equation (Figure 2). Considering patients' genotypes a tendency of lower CL/F was observed in patients with *CYP1A2*1F* CC genotype, compared to AA, while CA was intermediate. The frequency of *CYP1A2*1J* allele was very low and its influence on CL/F could not be evaluated. A significant trend of higher CL/F was observed in patients who were carriers of *CYP2C19*2* allele compared to other *CYP2C19* genotypes (Figure 3). Details of covariate model building are reported in Table 2. Univariate analysis of covariate relationships performed by forward inclusion into the base model revealed that A77 1726 CL/F is affected by GFR and presence of *CYP2C19*2* allele, while V/F is affected by patients weight, BSA and sex. Due to the high correlation between patient weight and BSA, only the former was introduced into the full model. In the backward elimination step, removal of the influence of patient weight on V/F from the full model resulted in non-significant increase in OFV of 2.707, corresponding to p = 0.10. Parameters of the final model are presented in Table 3.

The final model is described with the following equation:

$$CL/F\ [L/h] = 0.0374 \cdot 1.71^{gene} \cdot \left(\frac{GFR\ [ml/\min]}{110} \right)^{0.777}$$

$$V/F\ [L] = 7.66 \cdot 2.76^{sex}$$

Where *gene* = 1 if patient has a *CYP2C19*2* allele or 0 otherwise, and *sex* = 1 if patient is male.

In comparison to the base model, both inter-individual variability and proportional component of the residual variability decreased, while the additive component of the residual variability only slightly increased.

The exploration of diagnostic plots of population predicted versus observed concentrations, individual predicted versus observed concentrations and weighted residuals versus population predicted concentrations obtained from the base and final covariate model showed an improved fit of the final compared with the base model. However, the plots of population predicted versus observed concentrations showed a clear under-prediction at high A77 1726 concentration and over-prediction at low A77 1726 concentration (Figure 4).

When final NONMEM run was repeated with altered k_a, fixed at five times lower and five times greater value, parameter estimates of A77 1726 CL/F changed by less than 2% and estimates of the effect *CYP2C19*2* genotype ranged between 1.68 and 1.71, while the exponent on GFR ranged between 0.775 and 0.791. On the other hand, as expected the influence on estimation of V/F was more pronounced. Coefficient on V/F ranged from 4.36 to 7.84 L and the effect of sex on V/F ranged between 2.70 and 4.81. Alteration of k_a resulted in ΔOFV in the range between -0.786 and +14.429.

Average steady-state A77 1726 concentrations were higher in patients with ΔCRP of more than 8.5 mg/L (49.7 ± 39.0 mg/L) than in patients with ΔCRP of less or equal to 8.5 mg/L (24.8 ± 13.7 mg/L, p = 0.015, Figure 5). Similar non-significant trends were observed with other measurements of disease activity. A77 1726 C_{ss} was higher in patients with VAS of less or equal to 30 mm (45.3 ± 37.6 mg/L) than in patients with VAS of more than 30 mm (24.2 ± 13.4 mg/L, p = 0.057); C_{ss} was higher in patients with

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ΔESR of more than 5 mm/h (42.6 ± 40.0 mg/L) than in patients with ΔESR of less or equal to 5 mm/h (31.2 ± 18.2 mg/L, p = 0.967); C_{ss} was higher in patients with DAS28 less or equal to 4.3 (38.9 ± 36.7 mg/L) than in patients with DAS28 higher than 4.3 (31.9 ± 21.7 mg/L, p = 0.985).

At the inclusion of the study 5 patients had a record of ADRs. During the one year follow-up 5 additional patients developed severe ADRs and were discontinued from leflunomide. However, A77 1726 C_{ss} was not different in patients suffering from ADRs (34.7 ± 23.1 mg/L) compared to those reporting no ADRs (36.4 ± 31.9 mg/L, p = 0.682).

Discussion

The present study was the first that investigated the influence of genetic polymorphisms in cytochromes P450 on A77 1726 pharmacokinetics in relationship with leflunomide treatment response. Using a population pharmacokinetic model of A77 1726 we report on a novel data of the association of *CYP2C19*2* allele with A77 1726 steady-state plasma concentrations. We also confirm the previously reported association of A77 1726 steady-state plasma concentrations and leflunomide treatment response.

The pharmacokinetics of leflunomide has been studied in healthy adults as well as paediatric and adult patients with RA (Weinblatt et al., 1999; Li et al., 2002; Rozman, 2002; Chan et al., 2005; Shi et al., 2005). Large inter-individual variability in leflunomide pharmacokinetics was reported, with A77 1726 steady-state plasma concentrations ranging from 3 to 176 mg/L (Schmidt et al., 2003; van Roon et al., 2004; Chan et al., 2005; Shi et al., 2005; van Roon et al., 2005), and was also observed in our study. Previous leflunomide population pharmacokinetic studies demonstrated that some of the

variability can be explained by variation in patient age, gender, body size, liver function and smoking status (Chan et al., 2005; Shi et al., 2005; van Roon et al., 2005). To our knowledge no study investigated the influence of genetic variation in leflunomide-metabolizing enzymes on leflunomide pharmacokinetics.

An *in vitro* study on human liver microsomes has suggested that CYP1A2, CYP2C19 and CYP3A4 may be involved in the conversion of leflunomide to A77 1726 (Kalgutkar et al., 2003). On the basis of these data we hypothesized that SNPs in *CYP1A2* and *CYP2C19* that modify the corresponding enzyme activity may influence leflunomide metabolism. We investigated the association of SNPs in *CYP1A2* and *CYP2C19* with A77 1726 pharmacokinetics, but we did not study the genetic variability in *CYP3A4* due to a low frequency of *CYP3A4* polymorphic alleles in Caucasians and a limited genotype-phenotype relationship (He et al., 2005). We showed an association of *CYP2C19*2* allele with leflunomide pharmacokinetics as carriers of *CYP2C19*2* allele had significantly higher A77 1726 oral clearance compared to non-carriers. However, no effect of the presence of *CYP2C19*17* allele was found. This result is in accordance with the observations of previous studies where the presence of *CYP2C19*2* allele had a more pronounced effect on the metabolism of other CYP2C19 substrates, such as escitalopram and omeprazole than the presence of *CYP2C19*17* allele (Ohlsson Rosenborg et al., 2008; Rudberg et al., 2008). Based on our observation of higher CL/F in carriers of *CYP2C19*2* allele with lower enzyme activity, we assume that metabolic transformation of leflunomide to A77 1726 is decreased, leading to incomplete bioavailability (F). Our results are in agreement with the observation that rifampicin, inducer of many drug transporters and CYPs, including CYP2C19 (Flockhart,

http://medicine.iupui.edu/flockhart/table.htm) was found to significantly increase A77 1726 area under the plasma concentration-time curve in patients co-treated with rifampicin compared to patients on monotherapy with leflunomide (Food and Drug Administration Center for Drug Evaluation and Research, http://www.accessdata.fda.gov/drugsatfda_docs/nda/98/20905_ARAVA_BIOPHARMR.PDF; Hoechst Marion Roussel, http://products.sanofi-aventis.us/arava/arava.pdf).

In addition to genetic factors the influence of various demographic and biochemical parameters on A77 1726 pharmacokinetics was studied. Besides *CYP2C19*2* allele, renal function exerted significant effect on A77 1726 CL/F. This finding is in agreement with the fact that approximately half of A77 1726 is eliminated in urine. Moreover, it is in accordance with the results of a previous study in 6 patients with chronic renal insufficiency (Hoechst Marion Roussel, http://products.sanofi-aventis.us/arava/arava.pdf). The free fraction of A77 1726 in these patients was almost doubled, but the mechanism of the increase was not explained. Volume of distribution of A77 1726 was influenced with patient's gender. Since most of the patients included in our study were female, this result should be interpreted with some caution. But nevertheless, it is in accordance with the previously reported modest increase in V/F of 18% in male as compared to female patients (Food and Drug Administration Center for Drug Evaluation and Research, http://www.accessdata.fda.gov/drugsatfda_docs/nda/98/20905_ARAVA_BIOPHARMR.PDF).

We have previously shown that SNPs in *CYP1A2* but not in *CYP2C19* are associated with the occurrence of leflunomide-induced ADRs in RA patients (Bohanec Grabar et al.,

2008). However, the biological mechanism of this observation was not clear. We suggested that SNPs in *CYP1A2* alter the rate of the leflunomide activation which leads to the variation in plasma A77 1726 concentrations that might be associated with leflunomide toxicity. In this study we investigated the influence of genetic polymorphisms in *CYP1A2* on leflunomide pharmacokinetics. We observed only a non-significant trend of lower A77 1726 oral clearance in patients with *CYP1A2*1F* CC genotype, which may be due to low number of these patients. In order to further evaluate this observation, additional studies on larger cohort of patients are needed. Moreover, *in vitro* studies on human liver microsomes with known *CYP1A2* and *CYP2C19* genotypes could confirm this mechanism.

Previous studies investigated the influence of A77 1726 steady-state plasma concentrations on leflunomide treatment response in RA and juvenile RA patients (Chan et al., 2005; Shi et al., 2005; van Roon et al., 2005). The response of leflunomide treatment was assessed using either a decrease in DAS28 (van Roon et al., 2005), the number of swollen and tender joints, patients assessment of morning stiffness, VAS and measurement of physical and mental health status at the point of sampling (Chan et al., 2005) or definition of improvement of juvenile RA (Shi et al., 2005). Despite using different disease activity measurements, they all reported on a better response of leflunomide treatment in patients with higher A77 1726 steady-state plasma concentrations. In our study we assessed the treatment response using a decrease in CRP and ESR as well as DAS28 and VAS at the point of enrolment. Our data correlate well with the results of previous studies, as we observed a trend of higher decrease of CRP in patients with higher A77 1726 steady-state plasma concentrations. We observed no

association of A77 1726 steady-state concentrations with leflunomide toxicity, which is in agreement with the data from a previous study (Chan et al., 2005).

Our results indicate that plasma concentrations of leflunomide metabolite are associated with the treatment response, but not with leflunomide-induced toxicity. Genetic variations in leflunomide metabolizing enzymes that influence A77 1726 steady-state plasma concentrations, such as *CYP2C19*2* allele, are therefore associated with the response of leflunomide treatment. On the other hand we could not confirm the association of *CYP1A2*1F* allele with A77 1726 oral clearance and also observed no association of A77 1726 average steady-state concentrations with leflunomide toxicity. Hence, further investigations are necessary to elucidate the mechanisms of the association of genetic variation in leflunomide metabolizing enzymes with toxicity of the treatment.

The limitation of our study was that two variables CRP and ESR, but not DAS and VAS were measured twice and the overtime change was calculated for these variables (ΔCRP, ΔESR) only. Leflunomide monotherapy was not a criterion for the selection of the patients, as our cohort included 31, 11 and 23 patients co-treated with NSAIDs, MTX and corticosteroids, respectively. Nevertheless, when the data on co-medication was included in the model no significant influence on CL/F was observed. Hence, our results were not biased by patient co-treatment. We reduced the bias of our results due to non-compliance by detecting outliers, which allowed us to exclude two patients previously suspected of poor drug compliance. The strength of our study was that all RA patients were treated at the same clinic by the clinicians with a lot of experience with leflunomide treatment (Mladenovic et al., 1995; Smolen et al., 1999; Rozman, 2002). All the patients were also recruited in a geographic area with an ethnically homogeneous population thus, our

results were not biased by genetic heterogeneity of the patients (Tiilikainen et al., 1997; Vidan-Jeras et al., 1998). However, our study included relatively small number of patients and for this reason our results need a confirmation in a larger prospective study.

Despite the marked inter-individual variability, large drop-out rate and a clear relationship between A77 1726 concentrations and treatment response optimal therapeutic range of A77 1726 has not been yet established in patients with RA. Current treatment guidelines do not support therapeutic drug monitoring and a fixed dosing regimen comprised of a loading dose of 100 mg for three days and a maintenance dose of 20 mg is prescribed in the majority of patients. These data indicate that there is a room for improvement in the clinical use of leflunomide. Population pharmacokinetics and pharmacogenetics might be introduced as a new tool in the clinical practice to increase the efficacy of leflunomide treatment. As our study is the first to date that addressed the association of genetic polymorphisms in the leflunomide-metabolizing genes with A77 1726 pharmacokinetics, our observations that *CYP2C19*2* allele influences A77 1726 steady-state plasma concentrations and leflunomide treatment response, need an independent confirmation in a study with a larger sample size of patients treated with leflunomide.

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References

Aklillu E, Carrillo JA, Makonnen E, Hellman K, Pitarque M, Bertilsson L and Ingelman-Sundberg M (2003) Genetic polymorphism of CYP1A2 in Ethiopians affecting induction and expression: characterization of novel haplotypes with single-nucleotide polymorphisms in intron 1. *Mol Pharmacol* **64:**659-669.

Bohanec Grabar P, Rozman B, Tomsic M, Suput D, Logar D and Dolzan V (2008) Genetic polymorphism of CYP1A2 and the toxicity of leflunomide treatment in rheumatoid arthritis patients. *Eur J Clin Pharmacol* **64:**871-876.

Chan V, Charles BG and Tett SE (2005) Population pharmacokinetics and association between A77 1726 plasma concentrations and disease activity measures following administration of leflunomide to people with rheumatoid arthritis. *Br J Clin Pharmacol* **60:**257-264.

De Morais SM, Wilkinson GR, Blaisdell J, Meyer UA, Nakamura K and Goldstein JA (1994a) Identification of a new genetic defect responsible for the polymorphism of (S)-mephenytoin metabolism in Japanese. *Mol Pharmacol* **46:**594-598.

De Morais SM, Wilkinson GR, Blaisdell J, Nakamura K, Meyer UA and Goldstein JA (1994b) The major genetic defect responsible for the polymorphism of S-mephenytoin metabolism in humans. *J Biol Chem* **269:**15419-15422.

Ferguson RJ, De Morais SM, Benhamou S, Bouchardy C, Blaisdell J, Ibeanu G, Wilkinson GR, Sarich TC, Wright JM, Dayer P and Goldstein JA (1998) A new genetic defect in human CYP2C19: mutation of the initiation codon is responsible for poor metabolism of S-mephenytoin. *J Pharmacol Exp Ther* **284:**356-361.

Flockhart DA (2007) Drug Interactions: Cytochrome P450 Drug Interaction Table. *Indiana University School of Medicine*. Last accessed: June 2009.

Food and Drug Administration Center for Drug Evaluation and Research. Clinical pharmacology and biopharmaceutics reviews. Arava (leflunomide tablets) Tablet. Last accessed: June 2009.

He P, Court MH, Greenblatt DJ and Von Moltke LL (2005) Genotype-phenotype associations of cytochrome P450 3A4 and 3A5 polymorphism with midazolam clearance in vivo. *Clin Pharmacol Ther* **77:**373-387.

Herman D, Dolzan V and Breskvar K (2003) Genetic polymorphism of cytochromes P450 2C9 and 2C19 in Slovenian population. *Zdrav Vestn* **72:**347-351.

Hoechst Marion Roussel. Prescribing information for Arava tablets. Last accessed: June 2009.

Kalgutkar AS, Nguyen HT, Vaz AD, Doan A, Dalvie DK, McLeod DG and Murray JC (2003) In vitro metabolism studies on the isoxazole ring scission in the anti-inflammatory agent lefluonomide to its active alpha-cyanoenol metabolite A771726: mechanistic similarities with the cytochrome P450-catalyzed dehydration of aldoximes. *Drug Metab Dispos* **31:**1240-1250.

Kunkel G and Cannon G (2006) Leflunomide in the treatment of rheumatoid arthritis. *Expert Rev Clin Immunol* **2:**17-31.

Levey AS, Coresh J, Greene T, Stevens LA, Zhang YL, Hendriksen S, Kusek JW and Van Lente F (2006) Using standardized serum creatinine values in the modification of diet in renal disease study equation for estimating glomerular filtration rate. *Ann Intern Med.* **145:**247-254.

Li J, Yao HW, Jin Y, Zhang YF, Li CY, Li YH and Xu SY (2002) Pharmacokinetics of leflunomide in Chinese healthy volunteers. *Acta Pharmacol Sin.* **23:**551-555.

Lindbom L, Pihlgren P and Jonsson N (2005) PsN-Toolkit-A collection of computer intensive statistical methods for non-linear mixed effect modeling using NONMEM. *Computer Methods and Programs in Biomedicine* **79:**241-257.

Mladenovic V, Domljan Z, Rozman B, Jajic I, Mihajlovic D, Dordevic J, Popovic M, Dimitrijevic M, Zivkovic M, Campion G and et al. (1995) Safety and effectiveness of leflunomide in the treatment of patients with active rheumatoid arthritis. Results of a randomized, placebo-controlled, phase II study. *Arthritis Rheum* **38:**1595-1603.

Mosteller RD (1987) Simplified calculation of body-surface area. *N Engl J Med.* **317:**1098.

Ohlsson Rosenborg S, Mwinyi J, Andersson M, Baldwin RM, Pedersen RS, Sim SC, Bertilsson L, Ingelman-Sundberg M and Eliasson E (2008) Kinetics of omeprazole and escitalopram in relation to the CYP2C19*17 allele in healthy subjects. *Eur J Clin Pharmacol* **64:**1175-1179.

Rozman B (2002) Clinical pharmacokinetics of leflunomide. *Clin Pharmacokinet* **41:**421-430.

Rudberg I, Hermann M, Refsum H and Molden E (2008) Serum concentrations of sertraline and N-desmethyl sertraline in relation to CYP2C19 genotype in psychiatric patients. *Eur J Clin Pharmacol* **64:**1181-1188.

Sachse C, Brockmoller J, Bauer S and Roots I (1999) Functional significance of a C-->A polymorphism in intron 1 of the cytochrome P450 CYP1A2 gene tested with caffeine. *Br J Clin Pharmacol* **47:**445-449.

Schmidt A, Schwind B, Gillich M, Brune K and Hinz B (2003) Simultaneous determination of leflunomide and its active metabolite, A77 1726, in human plasma by high-performance liquid chromatography. *Biomed Chromatogr* **17:**276-281.

Shi J, Kovacs SJ, Wang Y, Ludden TM and Bhargava VO (2005) Population pharmacokinetics of the active metabolite of leflunomide in pediatric subjects with polyarticular course juvenile rheumatoid arthritis. *J Pharmacokinet Pharmacodyn* **32:**419-439.

Sim SC, Risinger C, Dahl ML, Aklillu E, Christensen M, Bertilsson L and Ingelman-Sundberg M (2006) A common novel CYP2C19 gene variant causes ultrarapid drug metabolism relevant for the drug response to proton pump inhibitors and antidepressants. *Clin Pharmacol Ther* **79:**103-113.

Skarke C, Kirchhof A, Geisslinger G and Lotsch J (2005) Rapid genotyping for relevant CYP1A2 alleles by pyrosequencing. *Eur J Clin Pharmacol* **61:**887-892.

Smolen JS, Kalden JR, Scott DL, Rozman B, Kvien TK, Larsen A, Loew-Friedrich I, Oed C and Rosenburg R (1999) Efficacy and safety of leflunomide compared with placebo and sulphasalazine in active rheumatoid arthritis: a double-blind, randomised, multicentre trial. European Leflunomide Study Group. *Lancet* **353:**259-266.

Tiilikainen A, Fischer G, Grubic Z, Gyodi E, Ivaskova E, Jungerman M, Kastelan A, Korinkova P, Martinovic I, Sochnevs A, Suciu-Foca N and Vidan-Jeras B (1997) Anthropological features of the East European region, in: *HLA: genetic diversity of HLA functional and medical implication. Proceedings of the 12th international histocompatibility workshop and conference* (Charron D ed), pp 307-313, EDK, Medical and scientific international publisher, Paris.

van Roon EN, Jansen TL, van de Laar MA, Janssen M, Yska JP, Keuper R, Houtman PM and Brouwers JR (2005) Therapeutic drug monitoring of A77 1726, the active metabolite of leflunomide: serum concentrations predict response to treatment in patients with rheumatoid arthritis. *Ann Rheum Dis* **64:**569-574.

van Roon EN, Yska JP, Raemaekers J, Jansen TL, van Wanrooy M and Brouwers JR (2004) A rapid and simple determination of A77 1726 in human serum by high-performance liquid chromatography and its application for optimization of leflunomide therapy. *J Pharm Biomed Anal* **36:**17-22.

Vidan-Jeras B, Jurca B, Dolzan V, Jeras M, Breskvar K and Bohinjec M (1998) Caucasian Slovenian normal, in: *HLA 1998* (Gjertson D and Terasaki P eds), pp 180-181, American society for histocompatibility and immunogenetics, Lenexa.

Weinblatt ME, Kremer JM, Coblyn JS, Maier AL, Helfgott SM, Morrell M, Byrne VM, Kaymakcian MV and Strand V (1999) Pharmacokinetics, safety, and efficacy of combination treatment with methotrexate and leflunomide in patients with active rheumatoid arthritis. *Arthritis Rheum.* **42:**1322-1328.

Footnotes

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Legends for figures

Figure 1. Chemical structures of leflunomide and its major metabolite A77 1726.

Figure 2. Influence of patients' glomerular filtration rate on A77 1726 oral clearance estimated with the base population model and loess fit (line).

Figure 3. Post-hoc estimates of individual patients' A77 1726 oral clearance (CL/F) with the base population model according to patient *CYP1A2* C-163A and *CYP2C19* genotypes. Horizontal lines indicate mean values in each genotype group.

Figure 4. Diagnostic plots of the final A77 1726 population pharmacokinetic model. Observed concentrations versus predicted concentrations based on the mean population model parameters (a) and predictions based on empirical Bayesian estimates (b) and weighted residuals versus population mean predictions (c). Line of identity (solid); regression line (dashed).

Figure 5. Average A77 1726 steady-state plasma concentration in relation to the decrease in CRP with leflunomide treatment ($p = 0.015$, Mann-Whitney U test).

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Tables

Table 1. Descriptive data for all study patients (N = 71).

Patient characteristic		N (%)
Demographic and clinical data		
Sex	male	14 (19.7)
	female	57 (80.3)
[a]Age [years]		59 (27-82)
[a]BMI		25.0 (16.5-34.1)
Current smokers		17 (23.9)
[a]Disease duration [months]		120 (7-480)
Presence of erosions		49 (75.4)
Treatment		
[a]Duration of leflunomide treatment [months]		35.8 (6.9-120.0)
Loading dose	100 mg/day	46 (68.7)
(first 3 days of treatment)	60 mg/day	12 (17.9)
Maintenance dose	20 mg/day	65 (91.5)
	10 mg/day	6 (8.5)
Co-treatment	NSAID	31 (43.1)
	MTX	11 (15.5)
	Corticosteroids	23 (31.9)
	Proton pump inhibitors	11 (15.5)

Biochemical assessments	
RF and/or anti-CCP seropositivity	55 (78.6)
[a]ALT [μkat/L]	0.35 (0.14-2.37)
[a]GFR [mL/min]	72.8 (30.3-106.4)
[a]ESR [mm/h]	22.0 (1.0-80.0)
CRP positivity (CRP ≥ 5 mg/L)	38 (54.3)
[a]Disease activity (DAS28)	4.3 (1.3-7.8)

[a]Continuous data expressed as median (range).

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Table 2. The distributions of *CYP1A2* and *CYP2C19* genotypes in RA patients.

Genotypes		Frequency n (%)
CYP1A2 C-163A	CC	4 (5.6%)
	CA	40 (56.3%)
	AA	27 (38.0%)
CYP1A2 C-729T*	CC	68 (98.5%)
	CT	1 (1.5%)
CYP1A2 T-739G*	TT	64 (94.1%)
	TG	4 (5.9%)
CYP2C19 genotypes	*1/*1	22 (31.0%)
	*1/*2	10 (14.1%)
	*1/*17	28 (39.4%)
	*17/*17	9 (12.7%)
	*2/*17	2 (2.8%)

*PCR amplification for *CYP1A2* C-729T and T-739G was unsuccessful in 2 and 3 patients, respectively.

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Table 3. Summary of the univariate analysis of covariate relationships.

Effect	ΔOFV	d.f.	p value	Estimate	s.e.
Clearance (CL/F)					
age	-0.774	1	0.379	-0.334	0.454
weight	-0.848	1	0.357	0.477	0.559
BSA	-0.956	1	0.328	0.875	0.956
sex	-0.150	1	0.699	0.914	0.217
smoking	-0.304	1	0.581	1.13	0.185
disease duration	-0.471	1	0.493	0.0615	0.0894
duration of leflunomide treatment	0.000	1	1.000	0.00127	0.00233
ALT	-0.032	1	0.858	-0.0341	0.172
GFR	-5.115	1	0.024	0.901	0.374
NSAID	-0.061	1	0.805	0.953	0.169
MTX	-0.306	1	0.580	1.15	0.283
corticosteroids	-1.097	1	0.295	1.23	0.228
Proton pump inhibitors	-0.121	1	0.728	0.915	0.171
[a]*CYP1A2* C-163A	-0.917	2	0.338	CA: 1.28	0.440
				AA: 1.44	0.519
[b]*CYP1A2* C-163A	-0.541	1	0.462	1.34	0.411
[c]CYP2C19	-7.415	4	0.006	**1/*2*: 1.89	0.476
				**1/*17*: 1.32	0.302
				**17/*17*: 0.945	0.229

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				**2/*17*: 2.36	0.421
[d]CYP2C19	-4.975	1	0.026	1.72	0.333
Volume of distribution (V/F)					
age	-1.075	1	0.300	0.366	0.227
weight	-6.606	1	0.010	1.62	0.544
BSA	-5.728	1	0.017	2.55	0.939
sex	-8.596	1	0.003	2.90	0.407
ALT	-1.706	1	0.192	0.366	0.371
NSAID	-0.991	1	0.319	1.34	0.429
MTX	-0.772	1	0.380	0.725	0.215
corticosteroids	-0.009	1	0.924	1.03	0.346
proton pump inhibitors	-0.395	1	0.530	0.794	0.208

ΔOFV – decrease of objective function value relative to the base model

d.f. – degrees of freedom

[a] Compared to CC genotype

[b] AA and CA combined against CC genotype

[c] Compared to **1/*1* genotype

[d] **1/*2* and **2/*17* combined against **1/*1*, **1/*17* and **17/*17*

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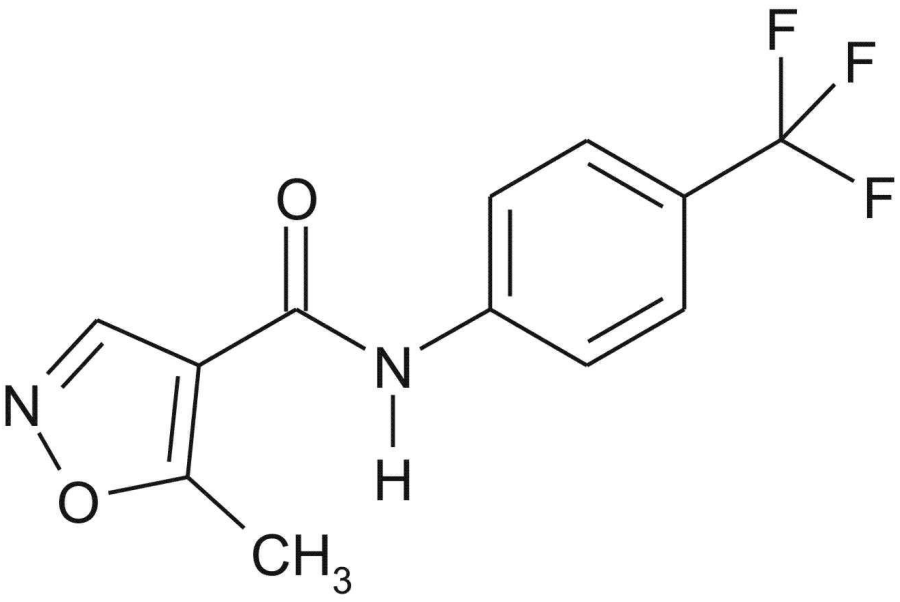
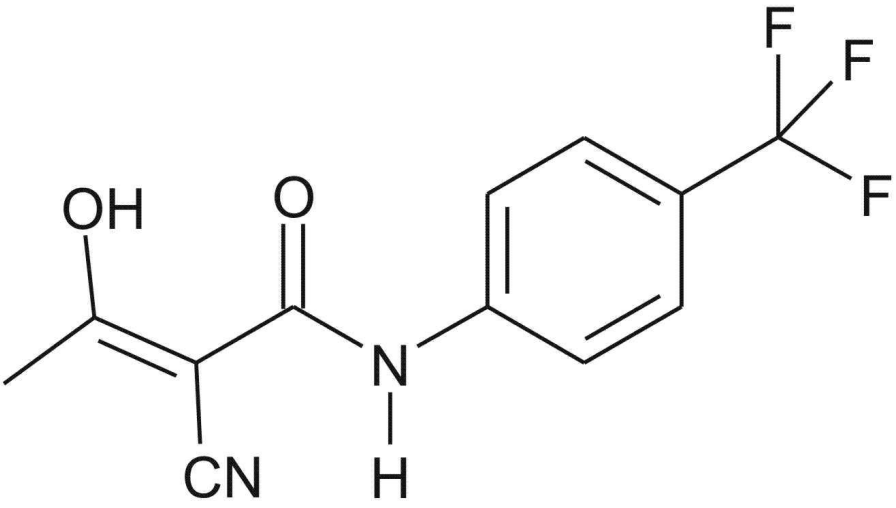
Table 4. Parameters of the final A77 1726 population pharmacokinetic model.

Parameter	Estimate	95% CI
Oral clearance		
CL/F [L/h]	0.0374	0.0235 – 0.0541
Effect of GFR	0.777	0.005 – 1.408
Effect of *CYP2C19*2* allele	1.71	1.25 – 2.63
Inter-individual variability [CV%]	73	58 – 86
Distribution volume		
V/F [L]	7.66	5.93 – 9.22
Effect of sex	2.76	1.67 – 5.80
Residual variability		
Proportional [%]	7.33	5.96 – 8.25
Additive [mg/L]	0.268	0.180 – 0.684

Fig. 1



Leflunomide



A77 1726

Fig. 2

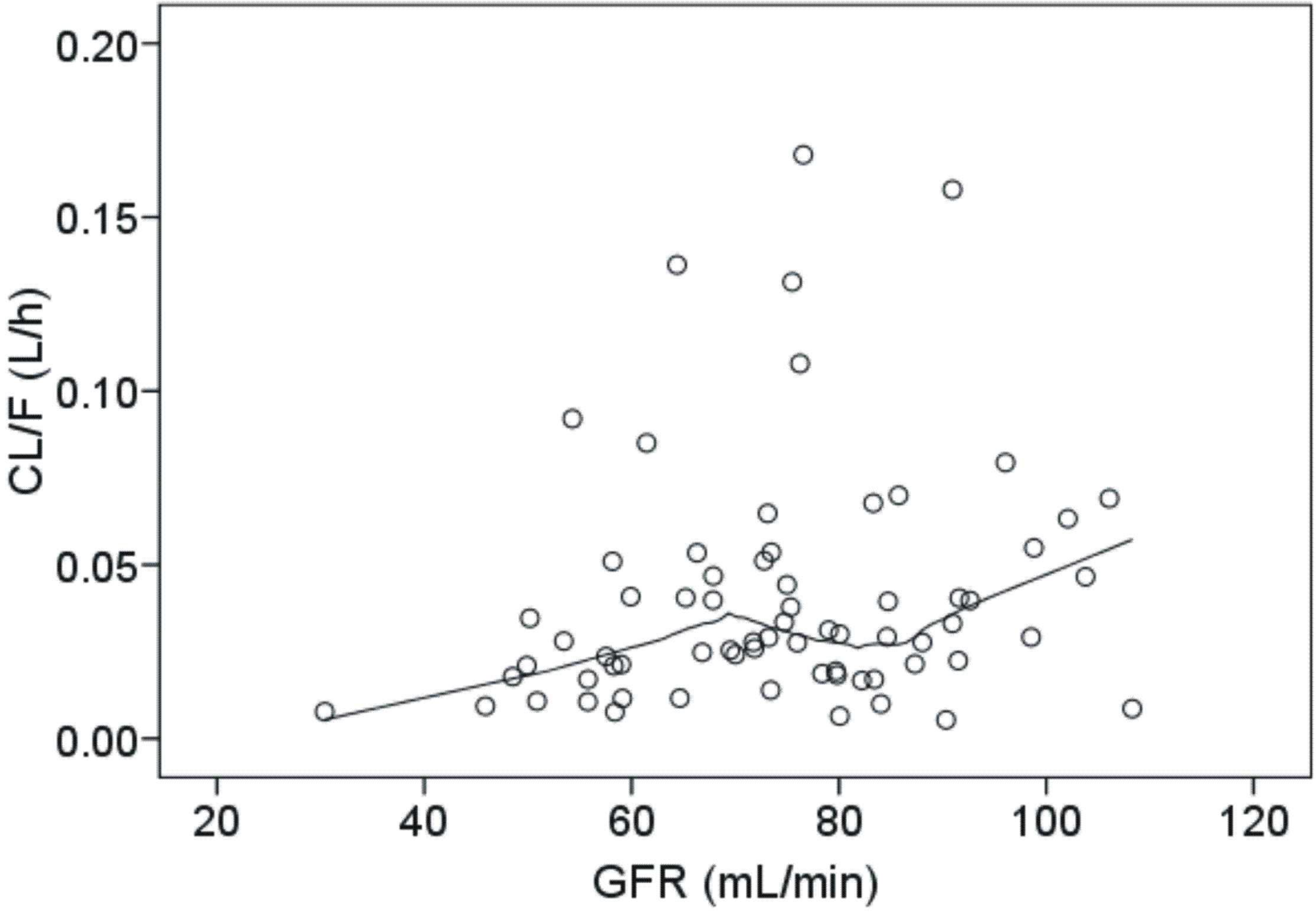
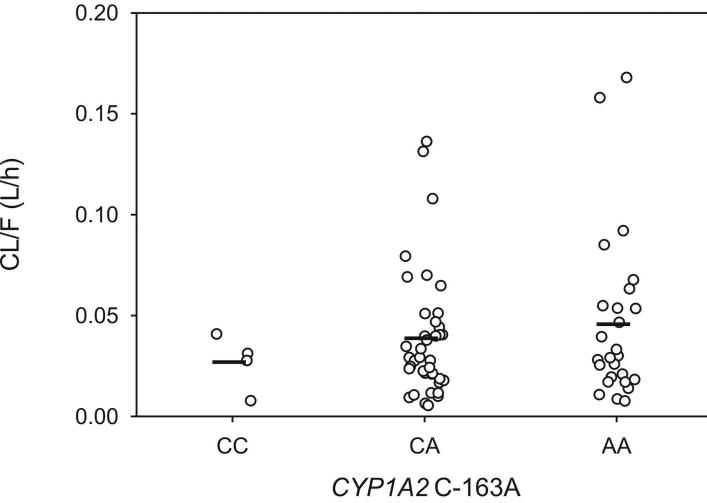


Fig. 3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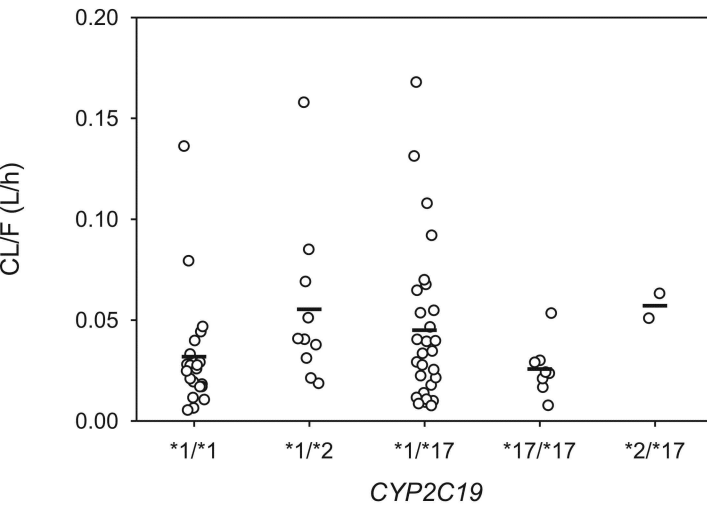
0.10
0.05
0.00
CL/F (L/h)
CC
CA
AA
CYP1A2 C-163A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0.15
0.10
0.05
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CL/F (L/h)
*1/*1
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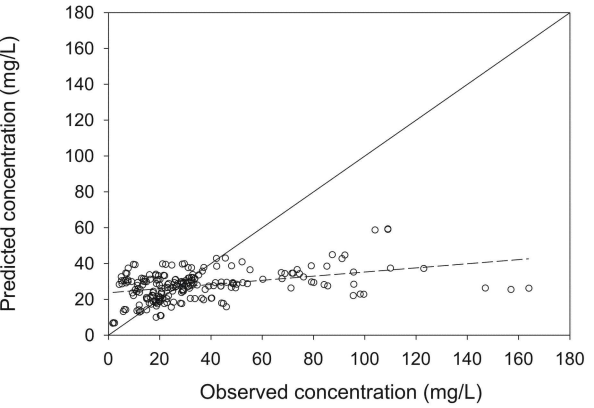
*17/*17
*2/*17
CYP2C19

Fig. 4

a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Observed concentration (mg/L)
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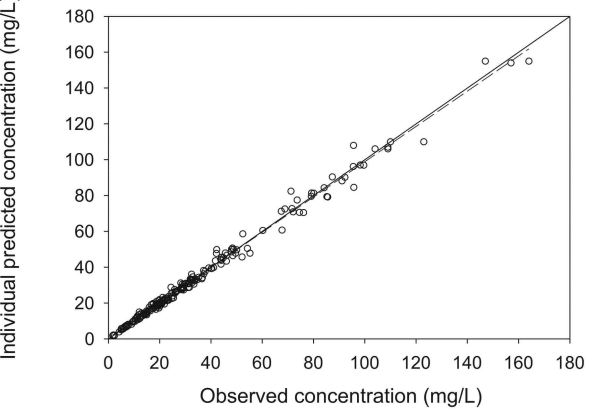
20
40
60
80
100
120
140
160
180

b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Observed concentration (mg/L)
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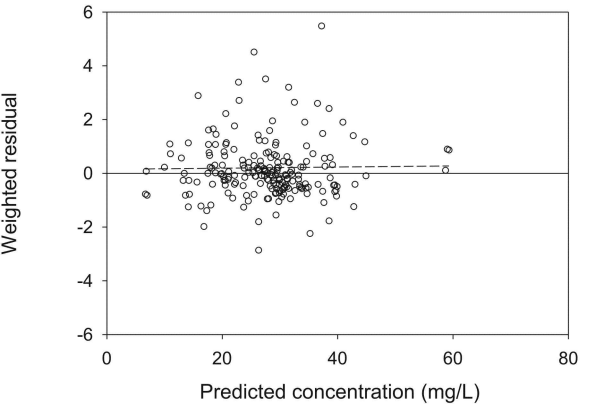
40
60
80
100
120
140
160
180

c


Weighted residual
Predicted concentration (mg/L)
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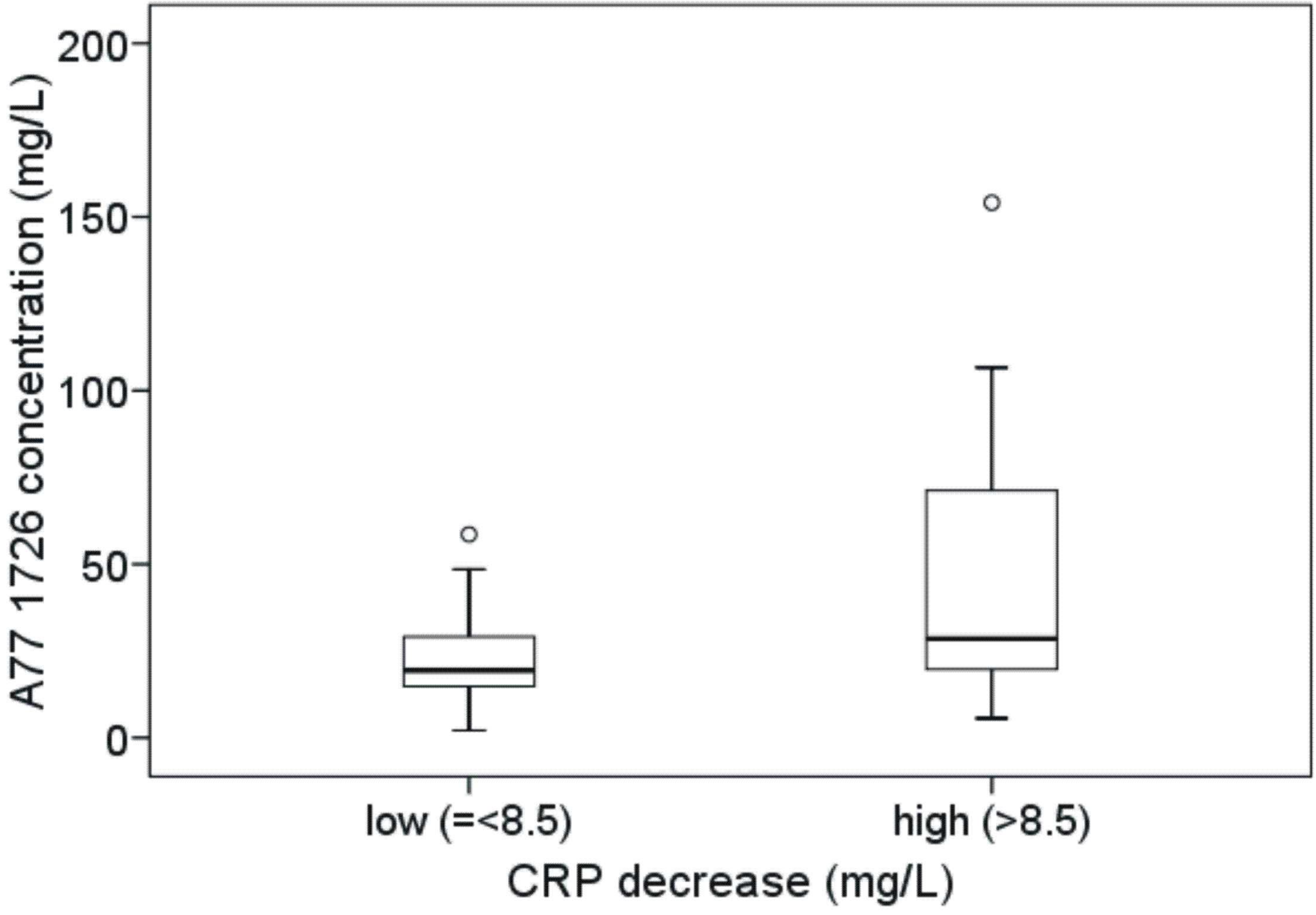
4
2
0
-2
-4
-6
0
20
40
60
80

Fig. 5


200
150
100
50
0
A77 1726 concentration (mg/L)
low (=<8.5)
high (>8.5)
CRP decrease (mg/L)