Genetic polymorphism of *CYP1A2* but not total or free teriflunomide concentrations is associated with leflunomide cessation in rheumatoid arthritis.

Running title: Time-to-event model of leflunomide cessation due to toxicity in RA patients

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Competing interests

All authors have completed the Unified Competing Interest form at http://www.icmje.org/coi_disclosure.pdf (available on request from the corresponding author) and declare: no support from any organisation for the submitted work; no financial relationships with any organisations that might have an interest in the submitted work in the previous 3 years; no other relationships or activities that could appear to have influenced the submitted work"

Summary

Aim(s)

Leflunomide, via its active metabolite teriflunomide, is used in rheumatoid arthritis (RA) treatment, yet approximately 20 to 40% of patients cease due to toxicity. The aim was to develop a time-to-event model describing leflunomide cessation due to toxicity within a clinical cohort and to investigate potential predictors of cessation such as total and free teriflunomide exposure and pharmacogenetic influences.

Methods

This study included individuals enrolled in the Early Arthritis inception cohort at the Royal Adelaide Hospital between 2000 and 2013 who received leflunomide. A time-to-event model in NONMEM was used to describe the time until leflunomide cessation and the influence of teriflunomide exposure and pharmacogenetic variants. Random censoring of individuals was simultaneously described. The clinical relevance of significant covariates was visualised via simulation.

Results

Data from 105 patients was analysed, with 34 ceasing due to toxicity. The baseline dropout hazard and baseline random censoring hazard were best described by step functions changing over discrete time intervals. No statistically significant associations with teriflunomide exposure metrics were identified. Of the screened covariates, carriers of the C allele of *CYP1A2* rs762551 had a 2.29 fold increase in cessation hazard compared to non-carriers (95% CI 2.24 - 2.34, p=0.016).

Conclusions

A time-to-event model described the time between leflunomide initiation and cessation due to side effects. The C allele of *CYP1A2* rs762551 was linked to increased leflunomide toxicity, while no association with teriflunomide exposure was identified. Future research should continue to investigate exposure-toxicity relationships, as well as potentially toxic metabolites.

What is already known about this subject

- Leflunomide is an effective treatment option in RA yet approximately 20 to 40% of patients fail due to toxicity.
- Teriflunomide's pharmacokinetics are highly variable, yet total concentrations have not been linked to toxicity, despite an association with response.
- Polymorphisms within *CYP1A2, CYP2C19* and *DHODH* have previously been linked to toxicity.

What this study adds

- A time-to-event model that included random censoring was developed that simulated cessation of leflunomide due to side effects in RA patients.
- Predicted total and free teriflunomide steady-state trough concentrations were not associated with toxicity.
- The instantaneous cessation hazard increased 2.29 fold in carriers of the C allele of *CYP1A2* rs762551.

Introduction

Leflunomide is a disease modifying antirheumatic drug (DMARD) used in the treatment of rheumatoid arthritis (RA), a chronic inflammatory autoimmune disease associated with severe morbidity, reduced functional ability and increased mortality[1]. Meta-analyses have shown that leflunomide has comparable efficacy to methotrexate, the gold standard for RA treatment[2, 3], and leflunomide is commonly used as a second line therapy option either as monotherapy or in combination with other conventional DMARDs in patients with resistant disease[4-6]. However, a major limitation for achieving remission with leflunomide is that up to 40% of patients discontinue therapy due to toxicity[2, 7, 8]. Gastrointestinal side effects (e.g. diarrhoea, nausea and vomiting; GI) are experienced by 20-30% of patients and are most likely to occur early in treatment and may settle if patients persist with therapy[8, 9]. In those who do not adequately respond to conventional DMARDs, biological DMARDs are considered[4-6]. In addition to being associated with an increased incidence of some cancers and serious infections[10], these agents are costly, so maximising remission rates with less expensive therapies such as leflunomide is likely to be a worthwhile strategy to maintaining patient outcomes whilst reducing the financial burden association with biological agents.

Cytochrome P-450 enzymes (CYP) -1A2, -2C19 and 3A4 are responsible for the metabolism of leflunomide to teriflunomide (the active metabolite), and previous studies have shown that genetic polymorphisms in *CYP1A2* and *CYP2C19* may be linked to increased toxicity[11, 12]. Furthermore, polymorphisms in *CYP2C19* have been associated with altered plasma concentration, but to date no association of concentration with *CYP1A2* genotype or smoking has been seen[13]. A single nucleotide polymorphism (SNP) within the first exon of dihydroorotate dehydrogenase *(DHODH,* rs3213422, 19C>A), the primary target of teriflunomide, has also been linked to increased toxicity[14]. Previous cross-sectional studies

have identified a relationship between total teriflunomide concentrations and leflunomide response in patients with RA, but no relationship with adverse drug events has been identified[13, 15, 16]. Plasma teriflunomide is highly bound to plasma proteins (>99%)[17], and for many drugs with a high degree of protein binding, total concentrations may be a poor surrogate of the free concentrations which are responsible for exerting clinical effects[18]. Studies have indicated variability between total and free teriflunomide concentrations[19, 20], but as yet free concentrations have not been investigated for a link to toxicity or response in RA patients.

The aim of this study was to develop a time-to-event model[21] describing the cessation of leflunomide due to toxicity, and to investigate potential predictors of this outcome including total and free teriflunomide exposure and pharmacogenetic markers.

Methods

Software

Modelling was performed using a Dell® Power Edge R910 server with 4x10 core Xeon 2.26 Ghz processors and 256 GB of RAM running Windows Server 2008 R2 Enterprise with a 64-bit operating system. Time-to-event model development was performed using NONMEM® Version VII Level 2.0 (Beal et al. 2009, ICON Dev. Soln., Ellicott City, MD) with the Wings for NONMEM (Version 720) interface (http://wfn.sourceforge.net/) and the G95 Fortran compiler.

Data manipulation, steady state concentration prediction, Kaplan-Meier analysis and survival model graphical output were conducted with the R Software Version 3.1.1 (R Core Team

2014) using the ggplot2, doBy, stringr, Hmisc, plyr, reshape2, gridExtra, scales, deSolve and survival packages[22-31].

Study population

Participants were aged ≥18 years, diagnosed with DMARD-naïve RA according to revised ACR Criteria[32] and enrolled in the RA inception cohort at the Royal Adelaide Hospital (RAH) between 2000 and 2013. Exclusion from the study included insufficient clinical records during the follow up period. All participants were treated according to a previously published treat-to-target protocol[6]. At diagnosis 'triple therapy' (methotrexate, sulfasalazine and hydroxychloroquine) was initiated. Patients were generally assessed every six weeks during active disease, or every three months once disease was inactive. If RA was active according to pre-defined criteria[6], sulfasalazine and then methotrexate were up-titrated to maximum tolerated doses. If this failed to control disease, leflunomide was added. During the first three years of the cohort study, leflunomide was initiated with a loading dose (three daily doses of 100 mg followed by 20 mg daily). Subsequently, this practice changed to an initial daily dose of 10 mg, and in the case of persistent disease, the daily dose was increased to 20 mg. Patients gave informed written consent for inclusion in the inception cohort and provision of a DNA sample. Ethics approval was obtained from the Human Research Ethics Committee of the RAH and the University of South Australia.

Event time data

Study participants were followed for up to 60 weeks after leflunomide initiation. Participants either i) continued to take leflunomide throughout the study period and were censored at the clinic visit date 'closest' to 52 weeks, or if the closest' visit date was greater than 52 weeks, were censored from the study at 52 weeks ii) had another DMARD added (due to persistent

disease activity) during the 60 week study period and were not considered to have ceased leflunomide due to side effects, but were censored at the date this new DMARD was added, or iii) ceased leflunomide due to side effects before 52 weeks. Treating clinicians were responsible for determining and recording the date of leflunomide cessation due to toxicity, with causality confirmed via a Naranjo categorisation of possible or greater[33].

At initiation of leflunomide, age, gender, smoking status, use of other DMARDs, leflunomide initiation dose and DAS28 were recorded, as was anti-cyclic citrullinated peptide antibody (anti-CCP), rheumatoid factor (RF) and shared epitope (SE) status at RA diagnosis. Missing values for continuous and categorical covariates were imputed with the median or mode respectively. At the individual's cessation or censor dates, height, weight, other DMARD doses, albumin, bilirubin, creatinine clearance (CrCl; Cockcroft-Gault Equation – IBW) and liver function test results were recorded. The fat free mass (FFM; based upon total body weight, height and sex[34]) was also calculated for individuals at these dates. Missing values for continuous and categorical covariates at the cessation or censor date were imputed, via forward inclusion, from the previous visit. *DHODH* haplotype, *DHODH* rs3213422 (C19A) genotype, *PTPN22* rs2476601 (C1858T) genotype, *CYP1A2* rs762551 (C163A) genotype, predicted *CYP2C19* 'phenotype' and *ABCG2* rs2231142 (C421A) genotype had all been determined prior to this study as described previously[12, 35, 36]. Predicted *CYP2C19* 'phenotype' was determined from rs4244285 and rs12248560 genotypes, and individuals were characterised as either unknown, ultra-rapid, extensive or poor and intermediate metabolisers, with unknown metabolisers imputed with the mode[12]. Haplotype, genotype or 'phenotype' grouping was conducted in the event of low allele frequencies (i.e. carriers of a homozygous recessive genotype with a frequency <10% were grouped with the heterozygous genotype).

Exposure metrics

Between 2010 and 2013, 69 participants involved in this study additionally gave informed written consent for collection of blood samples used to determine total and free teriflunomide concentrations, as previously described by Rakhila *et al.*[20]. Participants on a stable dose of leflunomide provided 2x10 mL blood samples collected at successive clinical visits (generally 6-12 weeks apart). Participants who initiated leflunomide between 2010 and 2013, had a blood sample collected before the first dose, followed by another at each of their next 5 clinic visits. All blood samples were collected as pre-dose trough samples.

For this data set, a semi-physiological pharmacokinetic model describing individual total and free teriflunomide concentrations, teriflunomide clearance (CLINT), volume of distribution (VBODY) and fraction unbound (FU) was developed[37]. From this model, empirical Bayes estimates of the physiological parameters controlling total and free teriflunomide concentrations were determined for each individual at their cessation or censor date. For individuals from whom no blood samples had been collected, physiological parameter estimates were imputed with the population median, as influenced by the individuals FFM and ALT concentrations[37]. Using the *posteriori* Bayes parameter estimates and the deSolve package of R, total and free teriflunomide steady state trough concentrations were predicted for each individual after 365 consecutive daily doses at the individual's average daily dose given across the study period. The average daily leflunomide dose across the study period and model predicted FU, CLINT and VBODY were also assessed as possible predictors of toxicity.

Structural model development

The time to leflunomide cessation due to toxicity was described by a time-to-event model developed in a stepwise manner where (i) a base model without any explanatory factors, apart from time, was built and (ii) the effect of potential covariates on the rate of leflunomide toxicity were investigated.

The likelihood of not having ceased leflunomide due to toxicity at time t, was described by the parametric survival function, S(t).

$$S(t) = e^{-\int_0^t h(t)dt} \qquad \text{Equation 1}$$

Where h(t) is the instantaneous hazard rate at time t, and S(t) is a function of the cumulative hazard function, H(t), between time zero and time t.

$$H(t) = \int_0^t h(t)dt \qquad \text{Equation 2}$$

As time-to-event models only use one observation for each individual, random effects on the baseline hazard could not be estimated, i.e. the same baseline hazard is assumed for all subjects. The probability density, f(t), of ceasing leflunomide at time t, was described by:

$$f(t) = S(t) * h(t) \qquad \text{Equation 3}$$

Base models were developed by exploring different functions for base hazards, $h_0(t)$, starting with a constant hazard, then Weibull, Gompertz, log-logistic distributions and finally, step functions. Selection of the base model was guided by visualisation of the estimated survival curve against a raw data Kaplan-Meier plot, as well as the change in Objective Function

Value (OBJ) as indicated by the lowest value of the Akaike's information criterion (AIC), where:

$$\mathrm{AIC} = \mathrm{OBJ} + 2 * \text{number of parameters} \qquad \text{Equation 4}$$

Equation 5 gives an example of a step function where the baseline hazard $h_0(t)$ changed depending on the time.

$$h_0(t) = \begin{cases} \theta 1, if\ 0 < t \leq t1 \\ \theta 2, if\ t1 < t \leq t2 \ldots \\ \theta n, if\ tn - 1 < t \leq tn \end{cases} \qquad \text{Equation 5}$$

A step function of this type was explored by starting with a single constant, and increasing the number of steps until no decrease in the AIC was observed. The time cut-offs for the steps were chosen based on the shape of a Kaplan-Meier plot of the raw data.

In order to facilitate simulation (see below), the random censoring of individuals before day 365 (e.g. cessation of leflunomide for reasons other than toxicity, having another DMARD added due to insufficient response or less than 365 days of follow-up) was also simultaneously described by a time-to-event model with its own $h_{0ran}(t)$, $S_{ran}(t)$, $H_{ran}(t)$ and $f_{ran}(t)$.

Covariate model development

Covariates were screened according to biological plausibility and prior knowledge of the factors considered to influence leflunomide toxicity. Covariates were included within functions (λ_{cov}) developed to modify the base hazard, $h_0(t)$, to give the hazard function, h(t):

$$h(t) = h_0(t) * \lambda_{cov1} * \ldots * \lambda_{covn} \qquad \text{Equation 6}$$

Categorical variants were assessed through a binary relationship (Equation 7), while continuous covariates were included as exponential functions normalised by the median covariate value (Equation 8). Concomitant drug effects were investigated via a linear function with their dose (Equation 9).

$$\lambda_{cov} = 1 + \theta * COV_{variant} \quad \text{Equation 7}$$

$$\lambda_{cov} = e^{\theta * (\frac{COV}{COV_{median}})} \quad \text{Equation 8}$$

$$\lambda_{cov} = 1 + \theta * (\frac{COV_{dose}}{COV_{median}}) \quad \text{Equation 9}$$

The potential inclusion of covariates on the base model were selected based upon a significant decrease in OBJ ($p<0.05$). The final model was developed via forward inclusion, where each covariate was sequentially added, starting with the covariate that caused the largest drop in the OBJ, and the following covariate(s) were only retained if the selection criteria were met.

Model evaluation and simulation

For the final structural and covariate models, nonparametric bootstrap analysis was used to assess the uncertainty of the parameter estimates. The original dataset was resampled 1000 times and analysed to estimate the 95% confidence intervals (95% CI) of the model parameters.

Time-to-event model visual predictive checks were used to assess the ability of the final structural and covariate models to describe the data[21, 38]. Using the R survival package, the survival probability of 1000 simulated replicate populations was computed at half daily intervals. The median and 95% CI of these survival probabilities were visually compared

against the Kaplan-Meier plot with 95% CI of the original dataset. To enable simulation, daily intervals from 1 to 365 were added to the original data for each individual. Covariate information was added to these additional records by a last observation carried forward/previous observation carried backward approach. The probability that an individual would be randomly censored (Equation 10) or cease leflunomide due to toxic effects (Equation 11) during an interval (i.e. a day) were respectively represented by conditional probabilities of failure, $S_{ran}(t_1^2)$ and $S(t_1^2)$:

$$S_{ran}(t_1^2) = \frac{S_{ran}(t_1) - S_{ran}(t_2)}{S_{ran}(t_1)} \qquad \text{Equation 10}$$

$$S(t_1^2) = \frac{S(t_1) - S(t_2)}{S(t_1)} \qquad \text{Equation 11}$$

In NONMEM, the conditional probabilities of random censoring and cessation were compared against two randomly generated uniform numbers between 0 and 1, representing the individual's probability of remaining in the study or ceasing leflunomide during that time period. If at time t_2, $S(t_1^2)$ was greater than the corresponding random number, the individual was said to have ceased leflunomide if they had not previously been lost to follow up due to a random censoring occurrence (i.e. had at a previous time $S_{ran}(t_1^2)$ been greater than its corresponding random number).

Results

Patients

A total of 115 patients commenced leflunomide and 10 were excluded due to incomplete records, leaving 105 participants available for analysis. Their baseline characteristics and teriflunomide exposure metrics are described in Table 1.

Of the 105 patients, 34 (32.4%) ceased leflunomide due to toxicity before day 365 of treatment. The side effects that contributed to the cessation of leflunomide are indicated in Table 2. Censoring from the study before day 365 occurred in 3 (2.9%) patients due to leflunomide cessation not related to toxicity, 9 (8.6%) due to inadequate response resulting in another DMARD being initiated, while in 33 (31.4%) patients, the closest clinic date to study completion was before day 365. The final clinic visit between day 365 and 420 occurred in 26 (24.8%) patients.

Two baseline DAS28 were missing from the data set and these were imputed with the median. RF, anti-CCP and SE status were imputed with the mode for one, two and four participants respectively, while *CYP2C19* phenotype[12] was unpredictable for eight individuals, who were similarly imputed with the mode. Blood samples were only available from 69 of the 105 participants, and thus participants with no samples had physiological parameter estimates that were imputed with the median as adjusted for the individuals ALT and FFM. These parameter estimates were used to predict the individuals total and free steady state trough teriflunomide concentration based upon their average leflunomide dose.

Structural model

The hazard functions $h_0(t)$ and $h_{0ran}(t)$ that best described the data with regards to AIC and visualisation against the original dataset Kaplan-Meier plot were step functions. For $h_0(t)$, results were best described by four time intervals, each with its own constant hazard rate, whereas $h_{0ran}(t)$ was best described by five time intervals. The resulting cessation hazard, $h_0(t)$, was high for the first 50 days, followed by a lesser decrease until day 112, after which it was higher until day 204, before another lesser decrease until the end of the study period. Conversely random censoring hazard, $h_{0ran}(t)$, increased significantly when comparing the first time interval against the final (Table 3).

Covariate model

Of the screened covariates, only *CYP1A2* C163A allele status met the inclusion criteria and no teriflunomide exposure covariates were identified (Table 4).

The 51 carriers of the C allele at *CYP1A2* C163A (i.e. CC or CA genotype) had a 2.29 fold (95% CI = 2.24 - 2.34) increase in cessation hazard compared to the 54 A homozygotes. The OBJ (1036.75) was 5.80 lower than with the structural model (p=0.016). Parameter estimates of the covariate model and the precision of these estimates are represented in Table 3.

Figure 1 (top) shows good concordance between the Kaplan-Meier plot of the observed population data against the median and associated 95% CI of the simulated survival data from the covariate model. Similarly, Figure 1 (bottom) shows good agreement between the observed and simulated data when facetted for carriers and non- carriers of the C allele of *CYP1A2* C163A, which was not observed for the original structural model (data not shown).

Discussion

A time-to-event model successfully described the time from leflunomide initiation until cessation due to toxicity in 105 RA patients taking leflunomide, most of whom were concurrently receiving other DMARDs. The cessation hazard was best described by a step function of four constants, while of 23 potential covariates, only *CYP1A2* C163A allele status significantly influenced the occurrence of toxicity.

The analysis showed cessation hazard was highest from days 0 to 50 and 112 to 204. No information was available that could explain this phenomenon, although it is possible that it was related to the scheduling of patients clinic visits. Intensive clinical follow-up occurred in patients with active disease compared to less frequent visits for those in remission, and there

was an overall tendency to see individuals at three and six months intervals, which corresponds to the periods of high dropout. Future research will aim to establish the external validity of the step functions, as despite describing the present population very well, the likelihood of describing an alternate population from a different recruitment site may be limited.

Previously, the CC genotype at *CYP1A2* C163A has been associated with a 9.7-fold increased risk of leflunomide cessation due to toxicity compared to the AC or AA genotype [p=0.002, odds ratio=9.708, 95%CI=2.276‑41.403][11]. Analysis of C allele carriers against non-carriers (i.e. AA vs AC + CC genotype) was conducted given the low CC genotype frequency (n=9; 8.6%) and previous research indicating that carriage of the C allele results in altered enzymatic effects[39]. In the present study carriers of one or more C alleles at *CYP1A2* C163A had a 2.29 fold increase in instantaneous cessation hazard compared to non-carriers (95%CI= 2.24 - 2.34, p=0.016). The C163A SNP is located in the promoter region of the CYP1A2 gene, and carriage of the A allele has been associated with enhanced metabolism of CYP1A2 substrates, particularly in cigarette smokers[11, 39]. However no association between smoking and leflunomide cessation was found. However, this is not surprising, as the rate of smoking (37.1%) in this study was small. Therefore as human liver microsome studies have suggested that CYP1A2, CYP2C19, and CYP3A4 are involved in the conversion of leflunomide to teriflunomide [40], carriers of the C allele may have lower teriflunomide concentrations. To date however, this has not been observed [13], which may be due to small sample sizes that have been unable to detect relatively small differences in teriflunomide concentrations that have high inter-patient variability, and/or CYP2C19 and CYP3A4 are able to maintain the high conversion of leflunomide to teriflunomide in individuals who carry a C allele at *CYP1A2* C163A. Furthermore, reduced CYP1A2 activity may promote altered metabolism that result in significant increases in alternate toxic metabolites that are ordinarily

at very low concentrations, or direct toxicity from leflunomide, both of which have been proposed previously[12, 19].

In this study, we aimed to investigate potential predictors of leflunomide toxicity with a major focus on total and free teriflunomide concentrations. Predicted steady state trough concentrations at the individuals' average dose across the study period was preferred over the actual teriflunomide concentration at drop-out, due to teriflunomide's long half-life (mean ~ 15.7 days), where approximately 8 weeks is required to achieve steady state[17, 41]. Instantaneous concentrations, particularly early in treatment and after dose changes, are highly time dependent and as such are imperfectly handled within time-to-event models, and are thus not a good metric to relate to toxicity between individuals on leflunomide for varying time lengths. Additionally, trough concentrations were used as they are subject to the influences of distribution effects, as well as clearance, which is the parameter solely responsible for the average steady state concentration. Therefore, predicted steady state trough concentrations were assessed, although to do this clinically, access to a population pharmacokinetic model, such as that used in this study[37], would be required. Despite this, no association was found, although we acknowledge that in this study, blood samples were only available from 69 of 105 participants, and given the high variability of teriflunomide concentrations, the population median (adjusted for the individuals ALT and FFM) is likely to underestimate the variability in individual concentrations within the 36 imputed subjects.

Previously, Chan *et al.*[16] and Grabar *et al.*[13] investigated a possible relationship between total steady-state teriflunomide concentrations and adverse drug events in 23 and 67 RA patients, respectively, and found no association. These studies however were cross sectional, with leflunomide mostly being used as monotherapy with some participants having been on leflunomide for long periods and therefore selecting individuals who were tolerant of

leflunomide. Furthermore, for many drugs free concentrations are more predictive of response and toxicity than total concentrations, particularly for those with a high degree of protein binding, such as teriflunomide, where the total concentration may be a poor surrogate of the free concentration[18, 20]. For the first time, predicted free steady-state teriflunomide concentrations have been investigated for a relationship with cessation of leflunomide due toxicity, although similar to previous studies, no association was found. Nor was an association found with the pharmacokinetic parameters (CLINT, VBODY or FU) predicted for each patient using our previously published model [37], or with the individual's average daily leflunomide dose across the study period.

In this study, multiple covariates were screened, of which *CYP1A2* C163A genotype was seen to result in a statistically significant effect on leflunomide cessation rate. In the occurrence of multiple covariates being included on forward inclusion, a backward deletion procedure ($p<0.005$) was planned for robustness of multiple hypothesis testing, although this was ultimately not possible given the single significant covariate that was identified. This study is not only the first study to assess the association between free teriflunomide concentration and leflunomide cessation, it is also the first to assess leflunomide cessation due to toxicity through the use of a time-to-event model within NONMEM. In contrast, previous studies have used binary logistic regression[11, 14], which assesses hazard at distinct time points rather than across time, or cox proportional hazard models which are restricted to assuming that hazard rates change proportionally with covariates across time[12]. This study used a time-to-event model and found a step function with changing hazard across time to best describe leflunomide cessation, while conducting the analysis within NONMEM allowed simulation of the effect of *CYP1A2* genotype, which has not been represented previously. Simulation highlights that although within our population the *CYP1A2* genotype effects on leflunomide cessation was large with parameter estimates that

are precise (%RSE of all parameters <2.15%), as the confidence intervals of the genotypes overlap, in some populations the effects of *CYP1A2* C163A may not be statistically significant with a sample size of 105 participants.

This study did not confirm the findings of a number of other studies, including that of Grabar *et al.*[14] who previously reported an association between *DHODH* rs3213422 genotype and toxicity. As previously described there are several differences between the presented cohort and that studied by Grabar *et al.*[14], who assessed toxicity through binary logistic regression. This study did not confirm the finding of an association between *CYP2C19* phenotype and cessation of leflunomide due to toxicity found by Wiese *et al.* [12], who assessed toxicity within the same cohort as used in this study (although the prior study finalised recruiting in 2011). In the present study, recruiting completed in 2013 and there were 105 eligible participants compared to the 78 assessed previously. This study also included longer follow-up in some participants, and identified some whom had ceased leflunomide but were restarted at a lower dose, hence explaining the moderately lower cessation rate. Furthermore, participants in this study with an undeterminable *CYP2C19* phenotype (7.6%) were imputed with the mode, in contrast these participant were previously excluded. Wiese *et al.*[12] used a cox proportional hazard model including covariates previously associated with leflunomide cessation, assuming a cessation trend was present ($p<0.25$; current leflunomide dose, triple therapy and positive rheumatoid factor at diagnosis) [12]. Furthermore, Wiese *et al.*[12] analysed the association with *CYP2C19* phenotype via a linear test for trend. The present study assessed cessation through a time-to-event model where covariates underwent a stricter inclusion criterion and categorical covariates were incorporated through a binary relationship.

Due to the observational nature of the study, the assessment of patient compliance was limited to reporting upon questioning at clinic visits, and although efforts were made to ensure all reported adverse events attributed to leflunomide were related and caused by leflunomide (as opposed to another DMARD or medication), certainty cannot be guaranteed. Also, due to the relatively small patient numbers and inability to precisely ascertain the relative severity of each side effect and, therefore the one primarily responsible for cessation, subgroup analysis of covariate effects on particular side effects was not conducted within this study.

In the present study, cessation was high early after leflunomide initiation, and due to teriflunomide's long half-life, plasma concentrations would be relatively low in patient ceasing early compared to those who continue therapy long term. Previous research has shown that individuals predominately cease leflunomide early due to GI side effects, especially with the use of the loading dose[42]; as such it appears likely these side effects are related to local effects rather than being linked to teriflunomide plasma concentrations. Therefore the subdivision of side effect appears important for future research as it may elucidate that side effects such as hepatotoxicity, pancytopenia and pneumonitis are related to high teriflunomide concentrations in the plasma, while the causes of GI side effects are related to local drug concentration not assessed in this study[8, 43-45]. Furthermore as *CYP1A2* genotype has now independently been indicated as a determinant of leflunomide toxicity, future studies should focus upon investigating previously proposed toxic metabolites [12, 19], that may be directly responsible for observable concentration dependent toxicity. Furthermore investigating alternate significant polymorphisms within the *CYP1A2* gene may elucidate different toxicity profiles within the leflunomide treated population, as the assessed polymorphisms within this study are not the only ones to be associated with altered enzymatic activity.

In conclusion, a time-to-event model describing time between leflunomide initiation and cessation due to side effects highlighted a significant association between *CYP1A2* C163A genotype and toxicity. And despite the large amount of random censoring which occurred, the simultaneous description of censoring hazard enabled the effect to be visualised via simulation. No association between the assessed teriflunomide exposure metrics and toxicity driven cessation was found; however given the missing the blood samples from a section of the cohort, future research should to continue to investigate the relationship, as well as characterising the impact of potentially toxic metabolites.

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Contributions of Authors

AMH, DJRF, RNU, MDW, CEO, and SMP were primarily responsible for study design. Data collection was primarily performed by AMH, MDW, CEO, and SMP. Data analysis was primarily performed by AMH, DJRF, RNU. All authors helped to draft the manuscript and have read and approved the final manuscript.

References

1. Scott DL, Wolfe F, Huizinga TWJ. Rheumatoid arthritis. The Lancet 2010; 376: 1094-108.

2. Osiri M, Shea B, Welch V, Suarez-Almazor Maria E, Strand V, Tugwell P, Wells George A. Leflunomide for the treatment of rheumatoid arthritis. In: Cochrane Database Syst Rev, Chichester, UK: John Wiley & Sons, Ltd, 2002.

3. Gaujoux-Viala C, Smolen JS, Landewé R, Dougados M, Kvien TK, Mola EM, Scholte-Voshaar M, van Riel P, Gossec L. Current evidence for the management of rheumatoid arthritis with synthetic disease-modifying antirheumatic drugs: a systematic literature review informing the EULAR recommendations for the management of rheumatoid arthritis. Ann Rheum Dis 2010; 69: 1004-09.

4. Singh JA, Furst DE, Bharat A, Curtis JR, Kavanaugh AF, Kremer JM, Moreland LW, O'Dell J, Winthrop KL, Beukelman T, Bridges SL, Chatham WW, Paulus HE, Suarez-almazor M, Bombardier C, Dougados M, Khanna D, King CM, Leong AL, Matteson EL, Schousboe JT, Moynihan E, Kolba KS, Jain A, Volkmann ER, Agrawal H, Bae S, Mudano AS, Patkar NM, Saag KG. 2012 Update of the 2008 American College of Rheumatology recommendations for the use of disease-modifying antirheumatic drugs and biologic agents in the treatment of rheumatoid arthritis. Arthritis Care Res 2012; 64: 625-39.

5. Smolen JS, Landewé R, Breedveld FC, Buch M, Burmester G, Dougados M, Emery P, Gaujoux-Viala C, Gossec L, Nam J, Ramiro S, Winthrop K, de Wit M, Aletaha D, Betteridge N, Bijlsma JWJ, Boers M, Buttgereit F, Combe B, Cutolo M, Damjanov N, Hazes JMW, Kouloumas M, Kvien TK, Mariette X, Pavelka K, van Riel PLCM, Rubbert-Roth A, Scholte-Voshaar M, Scott DL, Sokka-Isler T, Wong JB, van der Heijde D. EULAR recommendations for the management of rheumatoid arthritis with synthetic and biological disease-modifying antirheumatic drugs: 2013 update. Ann Rheum Dis 2014; 73: 492-509.

6. Proudman SM, Keen HI, Stamp LK, Lee ATY, Goldblatt F, Ayres OC, Rischmueller M, James MJ, Hill CL, Caughey GE, Cleland LG. Response-Driven Combination Therapy with Conventional Disease-Modifying Antirheumatic Drugs Can Achieve High Response Rates in Early Rheumatoid Arthritis with Minimal Glucocorticoid and Nonsteroidal Anti-Inflammatory Drug Use. Semin Arthritis Rheum 2007; 37: 99-111.

7. Aletaha D, Stamm T, Kapral T, Eberl G, Grisar J, Machold K, Smolen J. Survival and effectiveness of leflunomide compared with methotrexate and sulfasalazine in rheumatoid arthritis: a matched observational study. Br Med J 2003; 62: 944.

8. Alcorn N, Saunders S, Madhok R. Benefit-risk assessment of leflunomide: An appraisal of leflunomide in rheumatoid arthritis 10 years after licensing. Drug Saf 2009; 32: 1123-34.

9. van Riel PL, Smolen JS, Emery P, Kalden JR, Dougados M, Strand CV, Breedveld FC. Leflunomide: a manageable safety profile. The Journal of rheumatology 2004; 71: 21-24.

10. Furst D, Keystone E, Fleischmann R, Mease P, Breedveld F, Smolen J, Kalden J, Braun J, Bresnihan B, Burmester G. Updated consensus statement on biological agents for the treatment of rheumatic diseases, 2009. Ann Rheum Dis 2010; 69: i2-i29.

11. Grabar B, Rozman B, Tomši M, Šuput D, Logar D, Dolžan V. Genetic polymorphism of CYP1A2 and the toxicity of leflunomide treatment in rheumatoid arthritis patients. Eur J Clin Pharmacol 2008; 64: 871-76.

12. Wiese M, Schnabl M, O'Doherty C, Spargo L, Sorich M, Cleland L, Proudman S. Polymorphisms in cytochrome P450 2C19 enzyme and cessation of leflunomide in patients with rheumatoid arthritis. Arthritis Res Ther 2012; 14: R163.

13. Grabar B. Investigation of the influence of CYP1A2 and CYP2C19 genetic polymorphism on A771726 pharmacokinetics in leflunomide treated patients with rheumatoid arthritis. Drug Metab Dispos 2009; 37: 2061 - 68.

14. Grabar PB, Rozman B, Logar D, Praprotnik S, Dolžan V. Dihydroorotate dehydrogenase polymorphism influences the toxicity of leflunomide treatment in patients with rheumatoid arthritis. Ann Rheum Dis 2009; 68: 1367-68.

15. Van Roon EN, Jansen TLTA, Van De Laar MAFJ, Janssen M, Yska JP, Keuper R, Houtman PM, Brouwers JRBJ. 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 2005; 64: 569-74.

16. Chan V, Charles B, Tett S. 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 2005; 60: 257.

17. Wiese MD, Rowland A, Polasek TM, Sorich MJ, O'Doherty C. Pharmacokinetic evaluation of teriflunomide for the treatment of multiple sclerosis. Expert Opin Drug Metab Toxicol 2013; 9: 1025 - 35.

18. Dasgupta A. Usefulness of monitoring free (unbound) concentrations of therapeutic drugs in patient management. Clin Chim Acta 2007; 377: 1-13.

19. Hopkins AM, O'Doherty CE, Foster DJ, Upton RN, Proudman SM, Wiese MD. Individualization of leflunomide dosing in rheumatoid arthritis patients. Personalized Medicine 2014; 11: 449-61.

20. Rakhila H, Rozek T, Hopkins A, Proudman S, Cleland L, James M, Wiese M. Quantitation of total and free teriflunomide (A77 1726) in human plasma by LC-MS/MS. J Pharm Biomed Anal 2011; 55: 325-31.

21. Holford N. A time to event tutorial for pharmacometricians. CPT: pharmacometrics & systems pharmacology 2013; 2: e43.

22. Wickham H. ggplot2: elegant graphics for data analysis: Springer New York; http://had.co.nz/ggplot2/book, 2009.

23. Højsgaard S, Halekoh U, Robison-Cox J, Wright K, Leidi AA. doBy: doBy - Groupwise summary statistics, LSmeans, general linear contrasts, various utilities: CRAN.R-project.org, 2014.

24. Wickham H. stringr: Make it easier to work with strings: CRAN.R-project.org, 2012.

25. Harrell-Jr FE. Hmisc: Harrell Miscellaneous: CRAN.R-project.org, 2014.

26. Wickham H. plyr - The Split-Apply-Combine Strategy for Data Analysis. Journal of Statistical Software 2011; 40: 1-29.

27. Wickham H. Reshaping Data with the {reshape} Package. Journal of Statistical Software 2007; 21: 1-20.

28. Auguie B. gridExtra: functions in Grid graphics: CRAN.R-project.org, 2012.

29. Wickham H. scales: Scale functions for graphics: CRAN.R-project.org, 2014.

30. Karline Soetaert, Thomas Petzoldt, Setzer RW. Solving Differential Equations in R: Package deSolve. Journal of Statistical Software 2010; 33: 1-25.

31. Therneau TM. A Package for Survival Analysis in S: CRAN.R-project.org, 2014.

32. Arnett F, Edworthy S, Bloch D, Mcshane D, Fries J, Cooper N, Healey L, Kaplan S, Liang M, Luthra H. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. Arthritis Rheum 2005; 31: 315-24.

33. Naranjo CA, Busto U, Sellers EM, Sandor P, Ruiz I, Roberts E, Janecek E, Domecq C, Greenblatt D. A method for estimating the probability of adverse drug reactions. Clin Pharmacol Ther 1981; 30: 239-45.

34. Janmahasatian S, Duffull SB, Ash S, Ward LC, Byrne NM, Green B. Quantification of lean bodyweight. Clin Pharmacokinet 2005; 44: 1051-65.

35. O'Doherty C, Schnabl M, Spargo L, Cleland LG, James M, Proudman SM, Wiese MD. Association of DHODH haplotype variants and response to leflunomide treatment in rheumatoid arthritis. Pharmacogenomics 2012; 13: 1427-34.

36. Hopkins AM, O'Doherty CE, Foster DJR, Suppiah V, Upton RN, Spargo LD, Cleland LG, Proudman SM, Wiese MD. The rheumatoid arthritis susceptibility polymorphism PTPN22 C1858T is not associated with leflunomide response or toxicity. J Clin Pharm Ther 2014; 39: 555-60.

37. Hopkins AM, Wiese MD, Proudman SM, O'Doherty CE, Foster DJR, Upton RN. Semiphysiologically Based Pharmacokinetic Model of Leflunomide Disposition in Rheumatoid Arthritis Patients. CPT: pharmacometrics & systems pharmacology 2015; 4: 362-71.

38. Frobel AK, Karlsson MO, Backman JT, Hoppu K, Qvist E, Seikku P, Jalanko H, Holmberg C, Keizer RJ, Fanta S. A time-to-event model for acute rejections in paediatric renal transplant recipients treated with ciclosporin A. Br J Clin Pharmacol 2013; 76: 603-15.

39. Sachse C, Brockmöller J, Bauer S, Roots I. Functional significance of a C→A polymorphism in intron 1 of the cytochrome P450 CYP1A2 gene tested with caffeine. Br J Clin Pharmacol 1999; 47: 445-49.

40. Kalgutkar A, Nguyen H, Vaz A, Doan A, Dalvie D, McLeod D, Murray J. In vitro metabolism studies on the isoxazole ring scission in the anti-inflammatory agent leflunomide to its active -cyanoenol metabolite A771726: mechanistic similarities with the cytochrome P450-catalyzed dehydration of aldoximes. Drug Metab Dispos 2003; 31: 1240.

41. Sanofi-Aventis. Clinical Pharmacology and Biopharmaceutics Review: Teriflunomide. FDA Centre for Drug Evaluation and Research; http://wwwaccessdatafdagov/drugsatfda_docs/nda/2012/202992Orig1s000ClinpharmRpdf 2011: 1-194.

42. Siva C, Eisen S, Shepherd R, Cunningham F, Fang M, Finch W, Salisbury D, Singh J, Stern R, Zarabadi S. Leflunomide use during the first 33 months after food and drug administration approval: experience with a national cohort of 3,325 patients. Arthritis Care Res 2003; 49: 745-51.

43. Richards BL, Spies J, McGill N, Richards GW, Vaile J, Bleasel JF, Youssef PP. Effect of leflunomide on the peripheral nerves in rheumatoid arthritis. Intern Med J 2007; 37: 101-07.

44. Savage RL, Highton J, Boyd IW, Chapman P. Pneumonitis associated with leflunomide: a profile of New Zealand and Australian reports. Intern Med J 2006; 36: 162-69.

45. McEwen J, Purcell PM, Hill RL, Calcino LJ, Riley CG. The incidence of pancytopenia in patients taking leflunomide alone or with methotrexate. Pharmacoepidemiology and Drug Safety 2007; 16: 65-73.

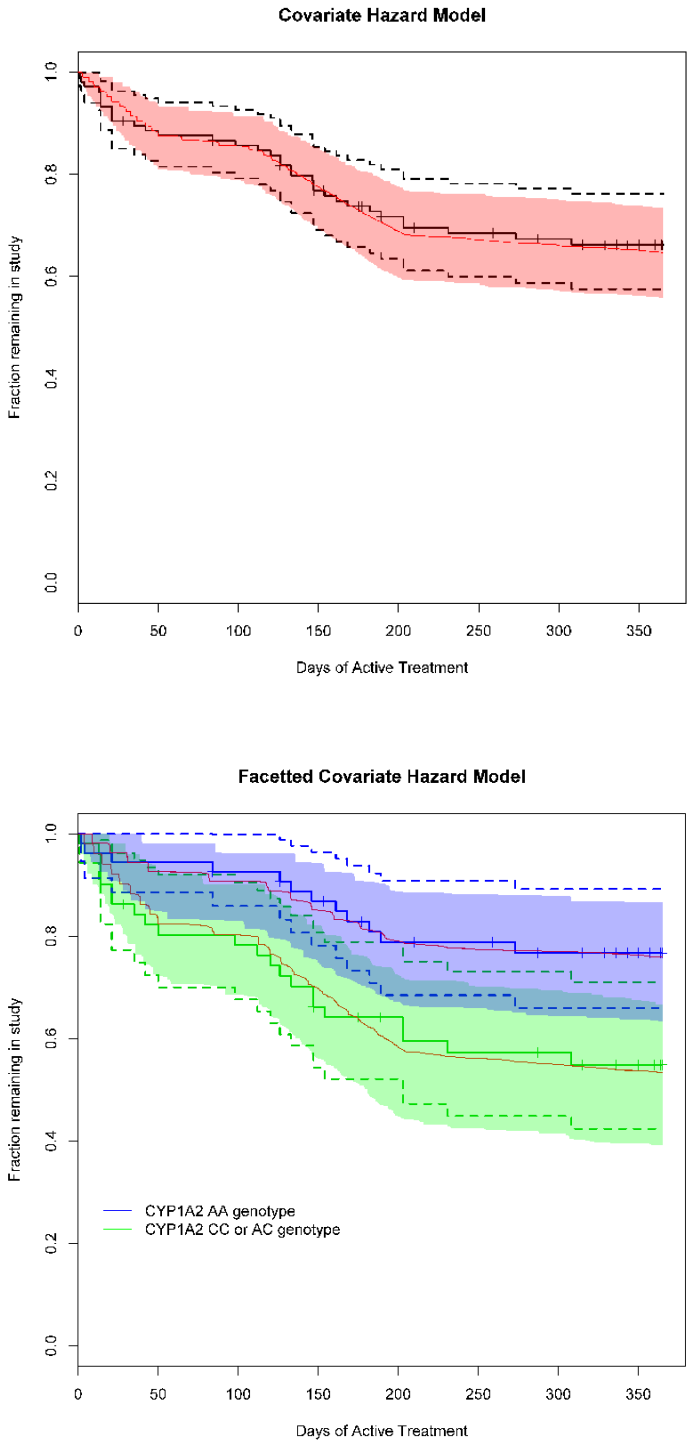

Figure 1: Top panel, Kaplan-Meier plot of the observed time-to-event data (black solid lines) and associated 95% CI (black dashed lines), overlaid with the 1000 simulated populations from the covariate models median (solid red line) and the associated 95% CI (shaded area). Bottom panel, the original dataset and the covariate models simulated populations have been facetted for *CYP1A2* C163A genotype.

Table 1: Characteristics of patients included in time-to-event analysis (n=105)

Patient characteristics at leflunomide initiation (n=105)	**n (% or range)**
Female	80 (76.2)
Age*	58.4 (19.2 – 85.9)
Current smoker	39 (37.1)
CYP1A2 C163A genotype	
- AA	54 (51.4)
- CC or AC	51 (48.6)
CYP2C19 phenotype[12]	
- Ultra-rapid metabolisers	26 (24.8)
- Extensive metabolisers	51 (48.6)
- Poor and intermediate metabolisers	20 (19.0)
- Unknown	8 (7.6)
ABCG2 rs2231142 genotype	
- CC	87 (82.9)
- AA or CA	18 (17.1)
DHODH rs3213422 genotype	
- AA	24 (22.9)
- AC	53 (50.5)
- CC	28 (26.7)
Carriers of *DHODH* Haplotype II	
- Yes	56 (53.3)
- No	49 (46.7)
DAS28* (n=103)	4.56 (1.48 – 7.98)
Initiated leflunomide with a loading dose	5 (4.8)
Initiated leflunomide while on MTX, SSZ and HCQ	59 (56.2)
Patient characteristics at RA diagnosis	
Anti-CCP positive (n=103)	60 (58.2)
RF positive (n=104)	67 (64.4)
SE positive (n=101)	71 (70.3)
Patient characteristics at cessation or censor date (n=105)	
Weight* (kg)	72.0 (40.4 – 148.5)
Height* (cm)	163 (145 – 194)
FFM* (kg)	44.4 (28.7 – 90.6)
Concomitant MTX use	80 (76.1)
- Weekly MTX dose* (mg)	20 (0 – 25)
Concomitant SSZ use	70 (66.7)
- Daily SSZ dose* (mg)	2000 (0 – 3000)
Concomitant HCQ use	91 (86.7)
- Daily HCQ dose* (mg)	400 (0 – 800)
ALT* (units/L)	24 (6 – 931)
AST* (units/L)	24 (11 – 432)
CrCl*(ml/min)	77.4 (20.6 – 197.0)
Exposure metrics (n=105)	
Daily leflunomide dose* (mg)	14.40 (6.64 – 100.00)
Predicted total teriflunomide steady state trough concentrations* (mg/L)	20.99 (1.09 – 135.21)
Predicted free teriflunomide steady state trough concentration* (mg/L)	0.0436 (0.0022 – 0.2879)
FU*	0.0021 (0.0014 – 0.0029)
CLINT* (L/day)	145.24 (6.55 – 2660.60)
VBODY* (L)	5.09 (2.49 – 19.87)

*data expressed as median (range).

Abbreviations – disease activity score in 28 joints (DAS28), anti–citrullinated protein antibody (Anti-CCP), rheumatoid factor (RF), shared epitope (SE), fat free mass (FFM), methotrexate (MTX), sulfasalazine (SSZ), hydroxychloroquine (HCQ), alanine transaminase (ALT), aspartate transaminase (AST), creatinine clearance (CrCl) and fraction unbound (FU).

Table 2: Toxicities leading to leflunomide cessation

Toxicity	Number of patients
Gastrointestinal (i.e. diarrhoea, nausea or vomiting)	20
Neutropenia	7
Fatigue, dizziness or headaches	5
Elevated liver enzymes	4
Respiratory related symptoms including pneumonitis	4
Other**	8

** One each of hair loss, peripheral neuropathy, muscle pain, giddiness, facial flushing, mouth ulcers, pulsating teeth and increased urinary frequency occurred.

Table 3: Parameter estimates (with bootstrap 95% CI and %RSE) for the structural and covariate model. The four cessation hazard constants are represented by $\Theta_{1\text{-}4}$ (days^{-1}); the five random censoring hazard constants are represented $\Theta_{5\text{-}9}$ (days^{-1}); and Θ_{10} represents the binary increase in λ_{CYP1A2} caused by the C allele of *CYP1A2* rs762551 (i.e. in carriers of the C allele of *CYP1A2* rs762551 $\lambda_{CYP1A2} = 1 + \theta_{10}$).

		Structural model			Covariate model		
Parameter		**Estimate**	**Bootstrap 95% CI**	**Bootstrap %RSE**	**Estimate**	**Bootstrap 95% CI**	**Bootstrap %RSE**
$h_0(t)$ (days^{-1})	**Time interval**						
Θ_1	**days < 50**	0.00232	0.00228 - 0.00237	0.98	0.00161	0.00158 - 0.00164	1.08
Θ_2	**50≥days<112**	0.00054	0.00052 - 0.00056	1.76	0.00034	0.00033 - 0.00035	2.12
Θ_3	**112≥days<204**	0.00234	0.00230 - 0.00238	0.83	0.00151	0.00148 - 0.00154	1.15
Θ_4	**days>204**	0.00034	0.00033 - 0.00035	1.75	0.00021	0.00020 - 0.00022	2.14
$h_{0ran}(t)$ (days^{-1})	**Time interval**						
Θ_5	**days<147**	0.00025	0.00024 - 0.00026	1.61	0.00024	0.00023 - 0.00025	1.73
Θ_6	**147≥days<210**	0.00134	0.00131 - 0.00138	1.35	0.00142	0.00138 - 0.00146	1.29
Θ_7	**210≥days<314**	0.00063	0.00061 - 0.00065	1.57	0.00068	0.00066 - 0.00070	1.49
Θ_8	**314≥days<350**	0.00768	0.00755 - 0.00781	0.87	0.00775	0.00762 - 0.00788	0.87
Θ_9	**days>350**	0.0302	0.02969 - 0.03071	0.86	0.0344	0.03392 - 0.03488	0.71
COV_{CYP1A2} (Θ_{10})		-	-	-	1.29	1.24 - 1.34	2.05

Table 4: Univariate covariate screening results

Model	ΔOBJ*	p-value
Base model	0	-
CYP1A2 C163A genotype (dichotomous)	-5.803	0.016
ALT*	-3.274	0.07
AST*	-2.849	0.091
Use of leflunomide loading dose	-2.784	0.095
Gender	-2.242	0.134
Average daily leflunomide dose*	-1.953	0.162
DHODH Haplotype II status (dichotomous)	-1.731	0.188
SSZ dose*	-1.295	0.255
Anti-CCP positivity	-1.164	0.281
DHODH rs3213422 genotype (polychotomous)	-0.862	0.353
CYP2C19 'phenotype' (polychotomous)	-1.653	0.438
Predicted free teriflunomide steady state trough concentrations*	-0.487	0.485
Age	-0.473	0.492
Triple therapy at initiation	-0.388	0.533
MTX dose*	-0.365	0.546
HCQ dose*	-0.326	0.568
RF positivity	-0.121	0.728

The covariates of baseline DAS28, SE status, weight, height, FFM, smoking status, ABCG2 genotype, CrCl, FU, CLINT, VBODY and predicted total teriflunomide steady state trough concentrations did not result in any improvement in the OBJ.

Abbreviations – alanine transaminase (ALT), aspartate transaminase (AST), sulfasalazine (SSZ), anti–citrullinated protein antibody (Anti-CCP, methotrexate (MTX), hydroxychloroquine (HCQ), rheumatoid factor (RF), disease activity score in 28 joints (DAS28), shared epitope (SE), fat free mass (FFM), creatinine clearance (CrCL) and fraction unbound (FU).

*- represent covariates that were measured at cessation or censor date