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## Optimizing study design in LPS challenge studies for quantifying drug induced inhibition of TNF $\alpha$ response: Did we miss the prime time?

Julia Larsson<sup>a,b,\*</sup>, Edmund Hoppe<sup>c,1</sup>, Michael Gautrois<sup>c</sup>, Marija Cvijovic<sup>b</sup>, Mats Jirstrand<sup>a</sup>

<sup>a</sup> Fraunhofer-Chalmers Centre, Chalmers Science Park, Gothenburg 412 88, Sweden

<sup>b</sup> Department of Mathematical Sciences, Chalmers University of Technology and University of Gothenburg, Gothenburg 412 96, Sweden

<sup>c</sup> Grünenthal GmbH, Aachen, Germany

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### ABSTRACT

In this work we evaluate the study design of LPS challenge experiments used for quantification of drug induced inhibition of TNF $\alpha$  response and provide general guidelines of how to improve the study design. Analysis of model simulated data, using a recently published TNF $\alpha$  turnover model, as well as the optimal design tool PopED have been used to find the optimal values of three key study design variables – time delay between drug and LPS administration, LPS dose, and sampling time points – that in turn could make the resulting TNF $\alpha$  response data more informative. Our findings suggest that the current rule of thumb for choosing the time delay should be reconsidered, and that the placement of the measurements after maximal TNF $\alpha$  response are crucial for the quality of the experiment. Furthermore, a literature study summarizing a wide range of published LPS challenge studies is provided, giving a broader perspective of how LPS challenge studies are usually conducted both in a preclinical and clinical setting.

### 1. Introduction

Tumor necrosis factor alpha (TNF $\alpha$ ) is a pro-inflammatory cytokine responsible for several immuno-responses and is involved in various signaling pathways in the body as well as in mediating inflammation (Jarosz-Griffiths et al., 2019). Due to its role in the pathogenesis of several immune-mediated diseases, it is considered an important biomarker for target engagement in the treatment against immune-mediated diseases, such as rheumatoid arthritis and Crohn's disease (Palladino et al., 2003). However, a difficulty with studying the response of TNF $\alpha$  to different interventions is that circulating TNF $\alpha$  is often undetectable in plasma of healthy organisms. To resolve this, pro-inflammatory challengers such as lipopolysaccharides (LPS) are used to induce cell activation and release of circulating TNF $\alpha$ .

Lipopolysaccharides (LPS) is a complex mixture derived from outer membranes of Gram-negative bacteria and is one of the most potent immune-stimulatory compounds (Munford, 2005). Exposure to LPS induces the release of several cytokines and a strong systemic inflammatory response similar to that observed in sepsis (Brooks et al., 2020; van Lier et al., 2019; Pfalzgraff et al., 2019). Due to this pronounced effect, experimental administration of exogenous LPS to preclinical animals is

frequently used to develop robust *in vivo* inflammation models for the identification of anti-inflammatory therapeutic drugs. Although LPS challenge models in a drug discovery setting do not intend to completely mimic inflammatory diseases in patients (Medzhitov, 2008), preclinical LPS challenge models in rodents and non-rodents have been shown to be very useful and are commonly used in drug discovery (Chakraborty et al., 2005; Gozzi et al., 1999; Shu et al., 2011; Wyska, 2010; Xiang et al., 2018). In addition, LPS challenge models can be used for translation of preclinical rodent models in early drug discovery as a proof-of-concept and target engagement studies in clinical trials (Brooks et al., 2020; van Lier et al., 2019).

Despite the frequent use, several technical difficulties need to be considered when conducting LPS challenge experiments. The difficulties arise from the fact that (a) a challenge by LPS causes a rapid and transient TNF $\alpha$  response that is over in a matter of hours, (b) that there is a lack of LC-MS/MS quantification methods sensitive enough to detect plasma concentrations of LPS, (c) undetectable TNF $\alpha$  baseline in absence of LPS, and (d) that comparison of large sets of compounds is confounded by high batch-to-batch variability and inter-individual variability typically seen with LPS. This in turn leads to uncertainties when distinguishing the stimulatory and inhibitory relationship

\* Corresponding author at: Fraunhofer-Chalmers Centre, Chalmers Science Park, Gothenburg 412 88, Sweden.

E-mail address: [julia.larsson@fcc.chalmers.se](mailto:julia.larsson@fcc.chalmers.se) (J. Larsson).

<sup>1</sup> Current address: Boehringer-Ingelheim GmbH & Co. KG, Drug Metabolism and Pharmacokinetics, Biberach a.d. Riss, Germany

between LPS provocation and test compound intervention when studying TNF $\alpha$  response data.

To better understand the relationship between the stimulatory effect of LPS and inhibitory effect of the drug, pharmacokinetic and pharmacodynamic modeling is used as a tool to understand the phenomena of LPS provocation despite the lack of complete information. Several examples of LPS challenge models exist (Chakraborty et al., 2005; Gabrielsson et al., 2015; Gozzi et al., 1999; Held et al., 2019; Hu et al., 2019; Wyska, 2010), where one of the most recent ones are described in Larsson et al. (2021). Here, an extensive data set was exploited, using multiple doses of both LPS and drug as well as including studies conducted at different occasions. The richness of information in the data gave the opportunity to create a complex and descriptive TNF $\alpha$  response model but using such a rich data set for every LPS challenge study would be infeasible and resource inefficient. A better approach is to utilize existing TNF $\alpha$  response models in order to make improvements to a reduced study design, which goes in line with the Three Rs (3Rs; Replacement, Reduction, and Refinement) as the guiding principle for more ethical use of animals in research testing (Russell and Burch, 1959).

In this work, we provide general guidelines of how to construct a study design for quantification of drug induced inhibition of TNF $\alpha$  response in LPS challenge studies using mathematical modeling. This is achieved by (1) optimizing important study design variables through mathematical modeling and model simulations, (2) comparing our suggestions with previously published study designs for validation of results, and (3) providing guidelines of how to translate LPS challenge studies from a preclinical to a clinical setting. The model and study design for the drug intervention considered in Larsson et al. (2021) serves as a case study and reference point. By using the recommendations provided in this study the information retrieved from an initial small pilot study should create sufficient insight to become a robust basis for designing future preclinical and clinical studies and trials in an

optimal way for drugs with similar properties. In addition, we believe that model informed study design helps avoiding 'try and error' and reduces unjustified animal usage to the best extent.

## 2. Theoretical development and methods

### 2.1. Design of a case study

In our previous work we developed a new TNF $\alpha$  modeling framework and quantified the pharmacodynamic effect of an orally administered drug, henceforth denoted Test Compound A (synthesized at Grünenthal, Aachen, Germany with batch purity of > 95%, full structure can be found in Supplementary material 1) (Larsson et al., 2021). In the drug intervention study, three different doses of the drug (0.3, 3, and 30 mg/kg) were given, together with one control (0 mg/kg, saline) to inbred Sprague-Dawley rats, as well as one intravenous dose of LPS (30  $\mu$ g/kg). Test Compound A was given 2 h before the LPS dose and measurements for the TNF $\alpha$  response were taken at 2, 2.5, 3, 3.5, 4, 5, and 6 h relative the drug administration (Fig. 1). Six rats were used per dose group giving a total of 24 rats in the study.

The presented case study serves as an example for illustration and provides start values of numerical and categorical design variables when optimizing the study design. The study design variables of interest are the sampling time points, the LPS dose, and the time delay between drug and LPS administration, as they serve as fundamental, important, and generic study design variables for LPS challenge studies in general. For instance, the lack of sustained TNF $\alpha$  response with sufficient duration can potentially result in large experimental errors due to the short duration for meaningful TNF $\alpha$  measurements. Thus, dynamic TNF $\alpha$  response can only be captured in a few observations during a short period of time, where the sampling time points should be chosen wisely. Secondly, the LPS dose needs to be sufficiently large such that the inhibiting drug effect is prominent despite the variability in response

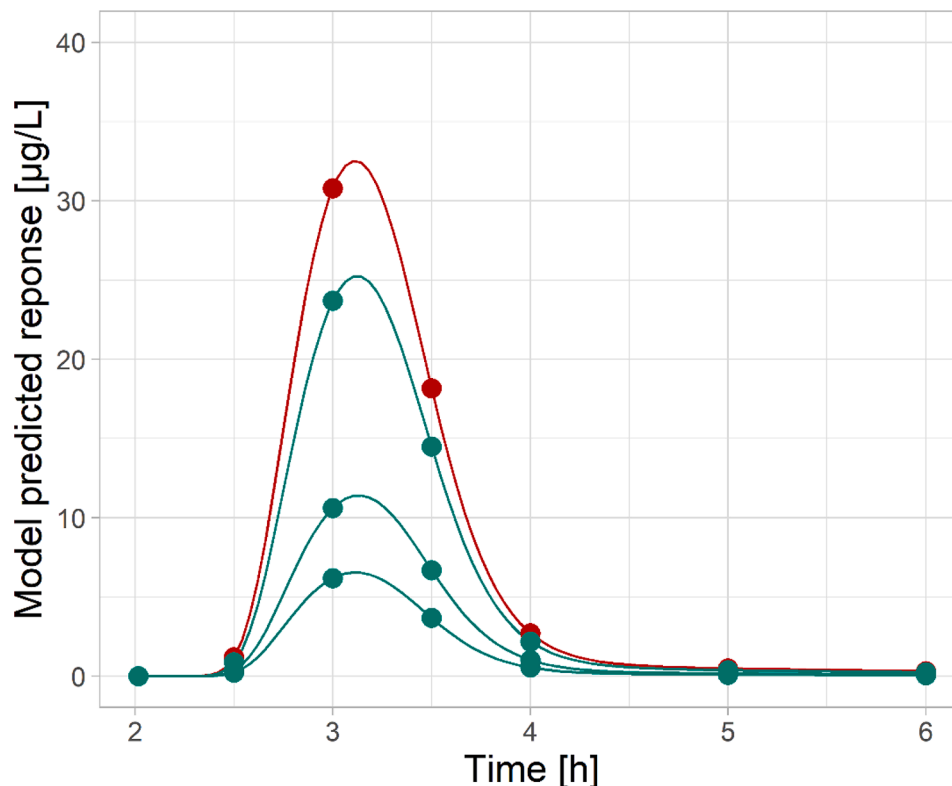


Fig. 1. Visualization of the TNF $\alpha$  response induced by an LPS challenge dose using the original study design where three drug doses were used (green lines) together with one vehicle control without drug (red line). The solid lines represent curve fits from the published model and the dots indicate the predicted values at the sampling time points in the original study design.

(Gabrielsson and Weiner, 2001) but simultaneously small enough to avoid adverse effects or intoxication by excessive cytokine release. Lastly, the choice of time delay between drug and LPS administration ( $\Delta t$ ) has previously been discussed as an important factor when it comes to TNF $\alpha$  inhibition (Almquist et al., 2020; van Eijk et al., 2014; Singh et al., 2015; Shu et al., 2011). The rule of thumb is usually that LPS should be administered when the drug reaches its maximum concentration, but since both the drug and TNF $\alpha$  response are transient, the resulting TNF $\alpha$  inhibition should be improved if  $\Delta t$  is chosen such that the onset, duration, and decline for both drug inhibition and LPS stimulation are matched.

The study design variables are optimized using statistical analysis of model simulations and the software PopED for optimal experiment design (Foracchia et al., 2004; Nyberg et al., 2012a). The specific model required for the model simulations and optimization is presented below.

## 2.2. Pharmacokinetic and pharmacodynamic models

The model used in this work consists of three main components: A TNF $\alpha$  turnover model, a pharmacokinetic model for Test Compound A, and a biophase model for the LPS provocation, where the two latter serve as inputs to the turnover model through inhibitory and stimulatory functions. This model has previously been developed using a rich data set using a non-linear mixed effects (NLME) approach. The model equations are repeated here for the convenience of the reader but for further information and details regarding the model development, see Larsson et al. (2021).

### 2.2.1. Exposure to test compound A

The oral dose of Test Compound A is modelled by a one-compartment model with first-order absorption from the gut into plasma, and the plasma exposure by a one-compartment model with first-order input and non-linear elimination (Eqs. (1) and (2))

$$\frac{dA_{ab}}{dt} = -k_a A_{ab}, \quad A_{ab}(t_0 - \Delta t) = D \quad (1)$$

$$V_p \frac{dC_p}{dt} = F \cdot k_a \cdot A_{ab} - \frac{V_{max} \cdot C_p}{K_m + C_p}, \quad C_p(0) = 0 \quad (2)$$

where  $A_{ab}$  denotes the amount in the gut and  $C_p$  denotes the plasma concentration of Test Compound A. The parameter  $k_a$  is the first-order absorption rate constant,  $D$  is the drug dose taken  $\Delta t$  hours before the LPS dose (where  $\Delta t$  is to be optimized),  $V_p$  is the volume of distribution,  $F$  is the bioavailability,  $V_{max}$  is the maximum rate of elimination, and  $K_m$  is the Michaelis-Menten constant.

### 2.2.2. Turnover of TNF $\alpha$ response after LPS and drugs

The stimulatory effect on TNF $\alpha$  response by LPS is described in Eqs. (3) and (4). A one-compartment disposition model of the LPS mixture with parallel first- and second-order loss of LPS was inferred from TNF $\alpha$  response-time data from the challenge experiments

$$\frac{dA_{LPS}}{dt} = -k_{LPS,1} A_{LPS} - k_{LPS,2} A_{LPS}^2, \quad A_{LPS}(t_0) = LPS_0 \quad (3)$$

where  $A_{LPS}$  denotes LPS amount in the biophase,  $LPS_0$  is the LPS dose given at time  $t_0=2$  h, and the parameters  $k_{LPS,1}$  and  $k_{LPS,2}$  are the first-order and second-order elimination constants of LPS. The level of LPS in plasma triggers an intra-cellular signaling cascade leading to TNF $\alpha$  release described as

$$\begin{aligned} \frac{dS_1}{dt} &= k_s \cdot \left( \frac{A_{LPS}}{K_{m,LPS} + A_{LPS}} - S_1 \right), \quad S_1(0) = 0 \\ \frac{dS_2}{dt} &= k_s \cdot (S_1 - S_2), \quad S_2(0) = 0 \\ \frac{dS_3}{dt} &= k_s \cdot (S_2 - S_3), \quad S_3(0) = 0 \end{aligned} \quad (4)$$

where  $S_1$ ,  $S_2$ , and  $S_3$  are unitless signaling entities and  $S_3$  acts on the build-up of TNF $\alpha$  response via stimulatory action. The parameter  $K_{m,LPS}$  is a half-maximal signal constant and  $k_s$  is a transduction constant inversely proportional to the delay time induced by the transduction compartments (Savic et al., 2007).

The TNF $\alpha$  turnover  $R$  and the impact of both the LPS challenge and the drug kinetics on the TNF $\alpha$  response is described in Eqs. (5) – (8). The stimulatory action of LPS  $S(S_3)$  on the TNF $\alpha$  release is described using a sigmoidal function

$$S(S_3) = \frac{S_{max} \cdot S_3^\gamma}{SC_{50} + S_3} \quad (5)$$

where  $S_{max}$  is the maximum LPS stimulatory production rate of TNF $\alpha$ ,  $SC_{50}$  is the quantity of  $S_3$  at 50% maximum stimulation, and  $\gamma$  the Hill coefficient. The inhibitory action  $I(C_p)$  of Test Compound A is described using an ordinary inhibitory  $I_{max}$  model

$$I(C_p) = 1 - \frac{I_{max} \cdot C_p}{IC_{50} + C_p} \quad (6)$$

where  $I_{max}$  is the drug efficacy and  $IC_{50}$  the drug potency. Eqs. (5) and (6) will then enter Eq. (7), where TNF $\alpha$  release is either stimulated (LPS exposure only) or simultaneously stimulated and inhibited (simultaneous LPS and drug exposure):

$$\frac{dR}{dt} = S(S_3) \cdot I(C_p) \cdot R - k_{out} \cdot R + k_t \cdot (R_t - R), \quad R(0) = 0 \quad (7)$$

$$\frac{dR_t}{dt} = k_t \cdot (R - R_t), \quad R_t(0) = 0 \quad (8)$$

The dynamics of TNF $\alpha$  response is divided into a central pool  $R$  and a peripheral pool  $R_t$  governed by a first-order inter-compartmental rate constant  $k_t$ . The irreversible loss of TNF $\alpha$  effect occurs from its central pool with fractional turnover rate constant  $k_{out}$ .

### 2.2.3. Data analysis

To add inter-individual variability, an NLME modeling approach is used. Specifically, inter-individual variability was given to  $V_{max}$  and  $k_{LPS,1}$  (Eqs. (2) and (3)) by assuming  $V_{max}$  normally distributed and  $k_{LPS,1}$  log-normally distributed with standard deviations  $\omega_{V_{max}}$  and  $\omega_{k_{LPS,1}}$ , respectively. Moreover, inter-occasion variability is not applied to  $k_{LPS,2}$  as in the original model since all data were conducted at the same occasion. Lastly, when simulating data from the TNF $\alpha$  turnover model, the concentration of  $R$  over time represents the TNF $\alpha$  response in plasma and the observation error is assumed to be proportional to  $R$  with standard deviation  $\sigma$ .

## 2.3. Optimization

### 2.3.1. Optimal time delay between LPS and drug administration

The optimal time delay between drug and LPS administration was investigated through systematic model evaluations and simulations, with the time delay and number of test subjects as the only variables. We tested if a noticeable change in TNF $\alpha$  response could be achieved by changing the time delay from its original value ( $\Delta t=2$  h) by comparing the ‘area under the effect curve’ of the model predicted TNF $\alpha$  response ( $AUC_{TNF\alpha}$ ) with neither inter-individual variability nor observation

error, for different time delays. After concluding that an improvement could be made, the cause of the improvement in terms of modelled drug inhibition and LPS stimulation was investigated (Eqs. (5)–(7)). When the optimal relationship in time delay between drug inhibition and LPS stimulation was found ( $\Delta t = \Delta t^*$ ), it was tested through model simulations if the improvement of TNF $\alpha$  response suppression would be significant after taking also the inter-individual variability and observation error into account.

Simulated TNF $\alpha$  response data for all three doses (0.3, 3, and 30 mg/kg Test Compound A) were retrieved using either the time delay from the original publication (2 h), or the optimized time delay derived from the median TNF $\alpha$  response curves ( $\Delta t^*$ ). The area under TNF $\alpha$  response curves ( $AUC_{TNF\alpha}$ ) from the simulated data was then calculated, square root-transformed to make the data normal distributed (since data showed negative skewness Rice, 2006), and compared using a two-sided *t*-test. As a measure of reliability, the confidence interval of each normally distributed group of  $AUC_{TNF\alpha}$  was calculated. Thus, except using the *p*-value as a test for significance, a second criterion was that these two confidence intervals should not overlap, to conclude that the compared groups do not come from the same distribution. The number of test subjects was successively changed up to 500 or until reliable statistical results were retrieved, while keeping the remaining parameters fix. The simulations were done in Mathematica using the package *NLMEModeling* (Leander et al., 2020, 2021), the statistical test was conducted in R, and the final result was visualized in R using the package *ggplot* (Wickham, 2016).

### 2.3.2. Optimal LPS dose and sampling time points

The optimal LPS dose and sampling time points were found using the optimal design tool PopED (Foracchia et al., 2004; Nyberg et al., 2012a), where the essential basis for the optimized planning of an experiment is provided by the Cramér-Rao inequality. The Cramér-Rao inequality states that the variance-covariance matrix of any unbiased estimator has a lower bound defined as

$$COV(q, y, \Theta) \geq FIM(q, \Theta)^{-1} \quad (9)$$

or, equivalently, that the difference between the variance-covariance matrix and the Fisher information matrix  $COV(q, y, \Theta) - FIM(q, \Theta)^{-1}$  is positive-semidefinite. Here *COV* is the variance-covariance matrix and *FIM* is the Fisher information matrix, a measure of the amount of information the data carries, defined as the negative expectation over data of the second derivative of the joint log likelihood dependent on the study design variables *q*, the observations *y*, and the model parameters  $\Theta$  (Foracchia et al., 2004; Nyberg et al., 2009, 2012a; Strömberg and Hooker, 2017). The parameters in  $\Theta$  are assumed to be fixed and the variables in *q* are the ones being optimized.

The goal in optimal design is to find the study design variables that gives the highest information content in data, quantified in terms of the Fisher information matrix. In practice, the size of the *FIM* is measured in terms of a design criterion that maps the matrix to scalar number, such that comparisons of different *FIMs* are plausible. PopED offers several design criteria that quantifies the size of the matrix differently. Here the default lnD-optimality was used and is defined as

$$OFV_{lnD} = \ln|FIM(q, \Theta)| \quad (10)$$

where  $OFV_{lnD}$  is the objective function and  $\ln|FIM|$  is the natural logarithm of the determinant of the Fisher information matrix (Nyberg et al., 2012a). Since the computation of the *FIM* is usually of high numerical complexity, there exist several options of how to approximate the *FIM*. In this work the full *FIM* and the first-order conditional estimation with interaction (FOCEI) was chosen, instead of the default settings. The full *FIM* was chosen to consider that parameters governing the inter-individual variability and the population parameters are correlated (Strömberg et al., 2016), and the FOCEI approximation to properly calculate the log likelihood of the model predicted TNF $\alpha$  response given

data, since the response differs considerably between subjects as a result of the inter-individual variability (Nyberg et al., 2012a). Furthermore, the parameters  $k_{LPS,2}$  and  $K_{m,LPS}$  were assumed fixed due to practical unidentifiability, since they both govern how the TNF $\alpha$  response changes for different LPS doses, which is not considered in this study design. Similarly, the pharmacokinetic parameters ( $k_a$ ,  $V_p$ ,  $F$ ,  $V_{max}$ ,  $K_m$ ,  $\omega_{Vmax}$ ) are fixed as they do not directly affect the TNF $\alpha$  response. For more details concerning model assumptions during study design optimization and alternative PopED settings, see Supplementary material 1. Apart from the approximation method of the *FIM* mentioned above the default settings were used, and for a complete list of available settings in PopED, see Nyberg et al. (2012a).

The sampling time points and LPS dose have been optimized simultaneously for improved results (Nyberg et al., 2009), using the values stated in Section 2.1 as a starting guess together with boundaries derived both from literature (Supplementary Material 2) and sensitivity analysis of the TNF $\alpha$  turnover model. To verify feasibility of the boundaries, using model simulations we tested if the median model predicted TNF $\alpha$  response was above the lower limit of quantification for the ELISA assay used in the original study (0.0125  $\mu$ g/L) (Larsson et al., 2021). Furthermore, we restricted the LPS dose and measurements to only take discrete values in the given range, for computational simplicity and practical feasibility. The LPS dose range is 3–300  $\mu$ g/kg but can only take the minimum value and then every tenth value (3, 10, 20, ..., 300). The sampling time points range between 0 and 6 h after drug administration with a minimum time difference of 20 min between measurements, which was the minimum difference observed in the literature (see (Held et al., 2019; Hu et al., 2019; Izeboud et al., 1999; Wang et al., 2007) and Supplementary Material 1 or 2). For a summary of fixed or optimized study design variables, see Table 1.

As a measure of design improvement from the original design, D-efficiency was used. D-efficiency is defined as

$$D_{eff} = \left( \frac{|FIM(q^*, \Theta)|}{|FIM(q, \Theta)|} \right)^{1/p} \quad (11)$$

where  $D_{eff}$  is the D-efficiency,  $|FIM|$  is the determinant of the Fisher information matrix for either the original study design variables *q* or the optimal variables  $q^*$ , and *p* is the number of parameters that is included in the optimization (i.e., not fixed).

As a last remark, the study design optimization results have been validated by testing the ability of retrieving the true parameters through estimation, which is used as a measure of practical identifiability. Specifically, ten parameter estimations in *NLMEModeling* with different initial guesses around the true parameter values were conducted, using the TNF $\alpha$  turnover model and simulated data from either the original or optimized study design (for details, see Supplementary material 1).

## 3. Results

### 3.1. Optimal time delay between LPS and drug administration

The median model predictions of TNF $\alpha$  response for varying time delays show that the apparent TNF $\alpha$  response suppression by Test

**Table 1**

Overview of the original experimental design that serves as a starting guess in the optimization, together with the corresponding boundaries.

Study design variable	Original value	Interval
Total number of test subjects	24	–
LPS dose [ $\mu$ g/kg]	30	[3, 300]
Number of dose groups (including control)	4	–
Drug doses [mg/kg]	(0, 0.3, 3, 30)	–
Sampling times relative drug dosing [h]	(2, 2.5, 3, 3.5, 4, 5, 6)	[2, 6]
Number of measurements	7	–
LPS administration relative drug [h]	2	–

Compound A is most effective if the time-point at maximal inhibition ( $I(C_p)$ , Eq. (6)) coincides with the time-point at maximal stimulation ( $S(S_3)$ , Eq. (5)) (Fig. 2a). Specifically, this happens when the time delay ( $\Delta t^*$ ) is 0.06, 0.09, and 0.3 h for the three drug doses, respectively. For the smallest and medium dose of Test Compound A (0.3 and 3 mg/kg) the difference is evident when compared to the TNF $\alpha$  response using the original time delay of 2 h, while for the largest drug dose (30 mg/kg) no visible difference in TNF $\alpha$  response was observed (Fig. 2b).

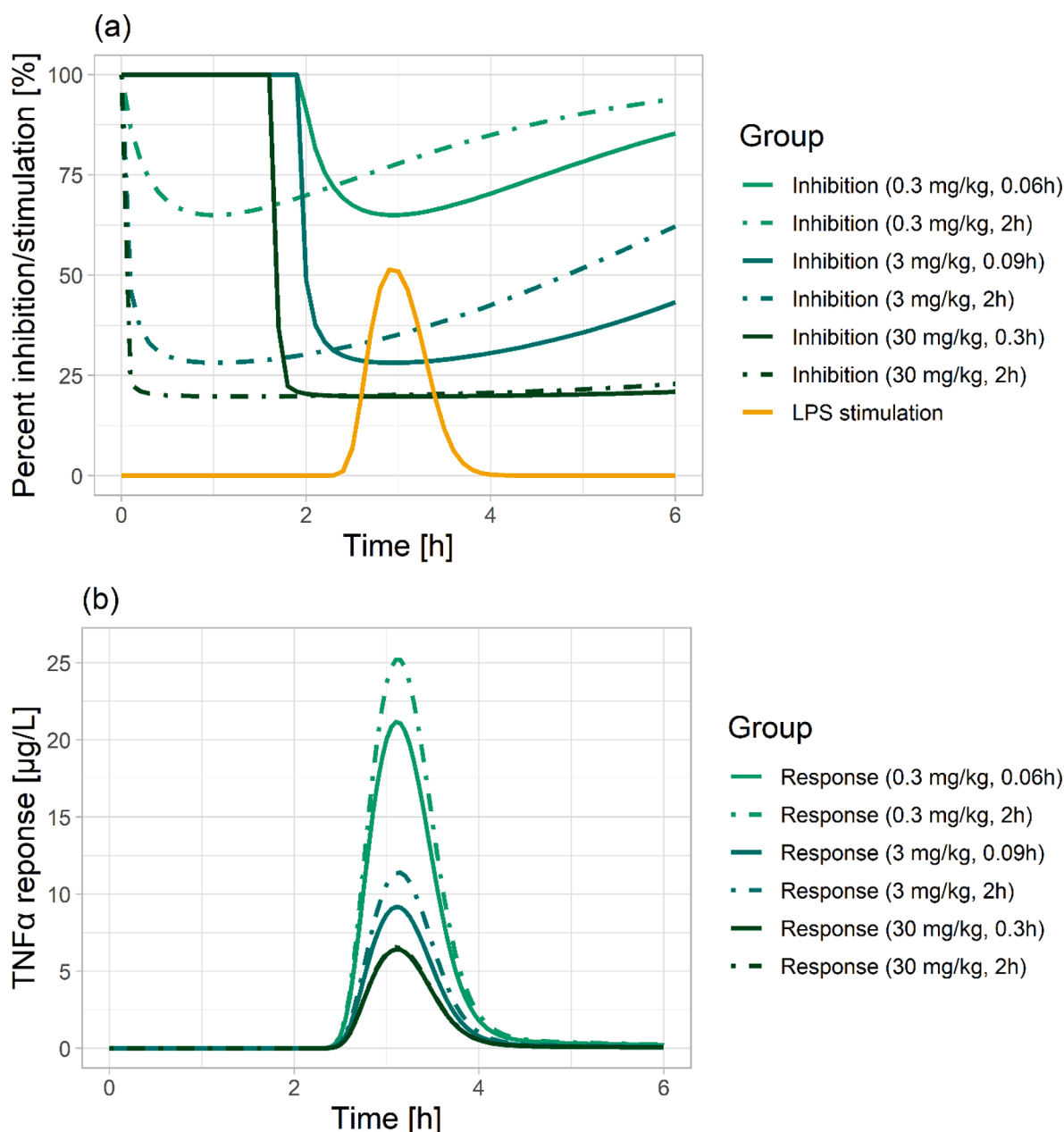
Comparing the area under the TNF $\alpha$  response curves ( $AUC_{TNF\alpha}$ ) for the median model predictions in Fig. 2 show that a difference of up to 20% in  $AUC_{TNF\alpha}$  can be achieved if the optimized time delay  $\Delta t^*$  (Table 2) is used. In addition, since a noticeable difference in  $AUC_{TNF\alpha}$  is only seen for the smallest and medium drug dose (0.3 and 3 mg/kg) whose value of  $\Delta t^*$  is approximately zero (0.06 and 0.09 h, or 3.6 and 5.4 min), the  $AUC_{TNF\alpha}$  using a ‘time delay’ of zero ( $\Delta t=0$  h, corresponding to the drug dose and LPS dose being taken simultaneously) is

**Table 2**

The area under the TNF $\alpha$  response curve ( $AUC_{TNF\alpha}$ ) for each dose group and different time delays ( $\Delta t$ ), as well as the change in  $AUC_{TNF\alpha}$  relative the original time delay of 2 h. The value of  $\Delta t^*$  for each dose group is 0.06, 0.09, and 0.3 h, respectively.

$\Delta t$ [h]	AUC [ $\mu\text{g/L}\cdot\text{h}$ ] per dose group			Relative AUC [%] per dose group		
	0.3 mg/ kg	3 mg/ kg	30 mg/ kg	0.3 mg/ kg	3 mg/ kg	30 mg/ kg
2	21.7	9.87	5.62	100	100	100
$\Delta t^*$	18.3	7.92	5.51	84.1	80.3	98.0
0	18.3	7.93	5.52	84.2	80.4	98.2

also presented. The  $AUC_{TNF\alpha}$  when using the simultaneous drug and LPS dosing is negligibly larger than when using the optimized time delay, implying slightly less effective TNF $\alpha$  suppression, but noticeably smaller than when using the original time delay of  $\Delta t=2$  h (Table 2). Therefore,

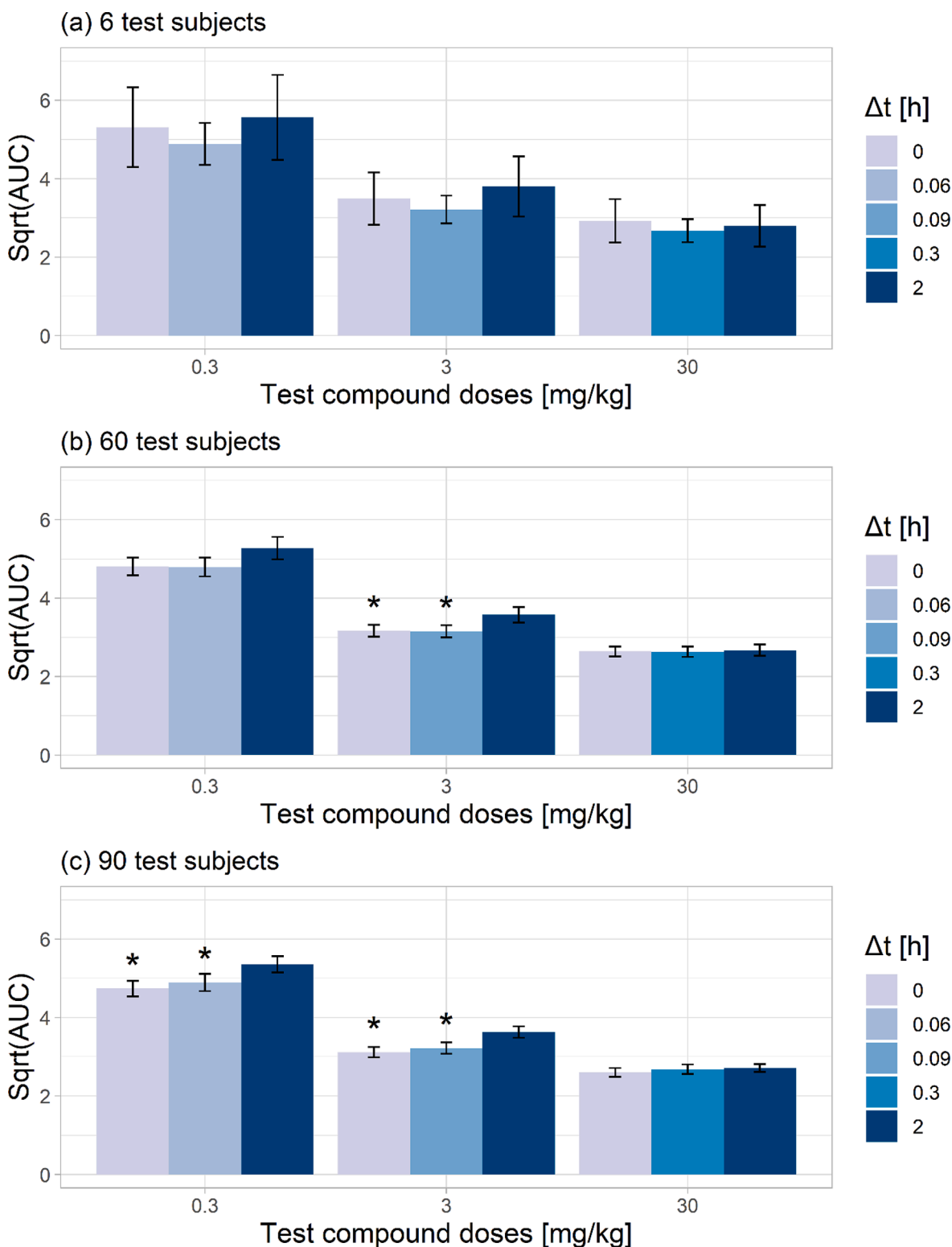


**Fig. 2.** (a) The inhibiting function (Eq. (6)) for the original time delay of  $\Delta t=2$  h and optimized time delay ( $\Delta t^*$ ), in relation to the normalized stimulation function serving as input to the TNF $\alpha$  response (Eq. (5)) divided by  $S_{max}$ . For the three doses of Test Compound A (0.3, 3, and 30 mg/kg),  $\Delta t^*$  is 0.06, 0.09, and 0.3 h, respectively. (b) The TNF $\alpha$  response using either the original (dot-dashed lines) or optimized (solid lines) time delay between drug and LPS administration.

the results from this analysis suggests that Test Compound A should be taken simultaneously with LPS, for reduced TNF $\alpha$  response and simplified study design when conducting the real-life experiments.

Although the median model predictions above show a clear difference in TNF $\alpha$  response, a significant difference in response is difficult to

demonstrate when adding the observation error and inter-individual variability to the model simulations (Fig. 3). The bar charts consist of transformed AUC<sub>TNF $\alpha$</sub>  for each dose group using either the original time delay ( $\Delta t = 2$  h) the optimized time delay ( $\Delta t^*$ , where  $\Delta t^*$  is 0.06, 0.09, and 0.3 h for each dose group), or the simultaneous dosing ( $\Delta t = 0$  h).



**Fig. 3.** Bar charts showing the square root transformed area under the TNF $\alpha$  response curve (AUC<sub>TNF $\alpha$</sub>  [ $\mu\text{g/L}\cdot\text{h}$ ]) for each dose group using either the original time delay ( $\Delta t = 2$  h), the optimized time delay ( $\Delta t^*$ , taking the value of 0.06, 0.09, or 0.3 h, dependent of dose group), or the simultaneous dosing ( $\Delta t = 0$  h), using (a) 6 test subjects, (b) 60 test subjects, or (c) 90 test subjects. The transformed AUC<sub>TNF $\alpha$</sub>  for the optimized time delay and the simultaneous dosing are compared with the transformed AUC<sub>TNF $\alpha$</sub>  for the original time delay using a  $t$ -test, and the significant difference between bar charts is visualized with a \* ( $p$ -value  $\ll 0.05$ ).

The normal distributed confidence intervals are shown for each bar chart and the asterisk (\*) indicates if the criteria defined in Section 2.3.1 have been achieved ( $p$ -value  $\ll 0.05$ ). No significant difference is seen for any of the dose groups using the number of test subjects from the original study design (Larsson et al., 2021), and instead approximately 60 and 90 test subjects are required to achieve a significant effect for the low and medium dose group, respectively. Although not shown, more than 500 test subjects were needed to retrieve significance for the largest dose group (30 mg/kg).

### 3.2. Optimal LPS dose and sampling time points

The results from the optimal design tool PopED show that the LPS dose should be increased from 30 to 260  $\mu\text{g}/\text{kg}$ , and the sampling time points in the elimination phase of TNF $\alpha$  response should be reconsidered (Fig. 4 and Table 3). The three first sampling time points before maximum TNF $\alpha$  response are identical in both designs, while the samples at the elimination phase are more frequently taken under a shorter time period for the optimized design. The two last measurements in the optimized design are suggested to be taken at the same time point (4.25 h).

The result in numbers can be found in Table 3, comparing both the study design variables as well as the parameter residual standard error (RSE) between the original and optimized design, where the RSE is calculated from the variance-covariance matrix of the model parameters. A decrease of RSE for almost all parameters is seen for the optimal design, especially for the parameters governing the production of TNF $\alpha$  response by LPS.

### 3.3. General guidelines for constructing LPS challenge studies and translation to humans

Previous sections served as a case study for showing specific improvements to a LPS challenge study design. Here the analysis of the results from the literature study are presented, giving a broader perspective considering LPS challenge studies in general in both a pre-clinical and clinical setting.

The measurements chosen by PopED are in concordance with what was found in the literature study (Fig. 5) and several similarities can be found between experiments in animals and humans (Table 4). The most prominent difference is the difference in duration of the experiment in animals versus humans, although there is good concordance for the measurements in the time interval 0–6 h relative LPS dosing (Fig. 5). In addition, the TNF $\alpha$  response data show very similar behavior between animals and humans according to the literature (Table 4), implying that the optimal sampling time points obtained in the section above could be

**Table 3**

The optimal study design retrieved from PopED with corresponding parameter residual standard error (RSE), in comparison with the original design.

Variable	Value, initial	Value, final	Difference
LPS dose [ $\mu\text{g}/\text{kg}$ ]	30	260	230
Sampling times [h]	(2, 2.5, 3, 3.5, 4, 5, 6)	(2, 2.5, 3, 3.5, 3.75, 4.25, 4.25)	(0, 0, 0, 0, -0.25, -0.75, -1.75)
$OFV_{MD}$	69.5	87.7	18.2
$D_{eff}$	–	5.19	–
Parameter residual standard error (RSE [%])			
$k_{LPS,1}$ [ $\text{h}^{-1}$ ]	28	9	-19
$k_s$ [ $\text{h}^{-1}$ ]	12	4	-8
$S_{max}$ [ $\mu\text{g}/\text{L}/\text{h}$ ]	19	16	-3
$SC_{50}$ [-]	25	5	-20
$\gamma$ [-]	1	0	-1
$k_{out}$ [ $\text{h}^{-1}$ ]	19	5	-14
$k_i$ [ $\text{h}^{-1}$ ]	19	6	-13
$I_{max}$ [-]	2	2	0
$IC_{50}$ [ $\mu\text{mol}/\text{L}$ ]	22	22	0
$\omega_{k_{LPS,1}}$ [-]	42	39	-3
$\sigma$ [-]	15	16	1

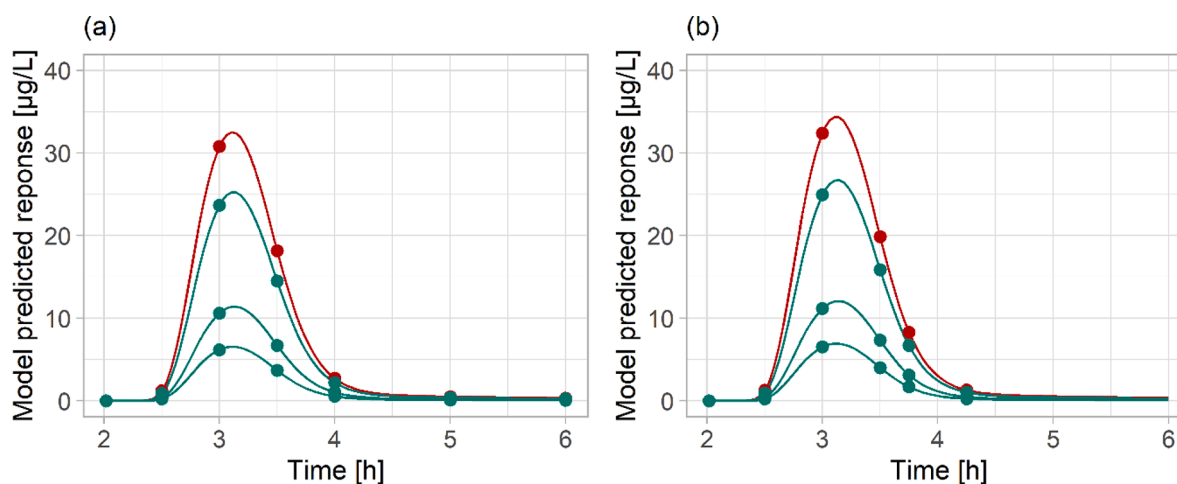
applied to clinical experiments as well. For a comparison of more study design variables, see Supplementary Material 1 and 2.

The general points provided here, serve as recommendations summarizing the key aspects to consider when planning LPS challenge studies, dependent of the purpose of the study (Table 5).

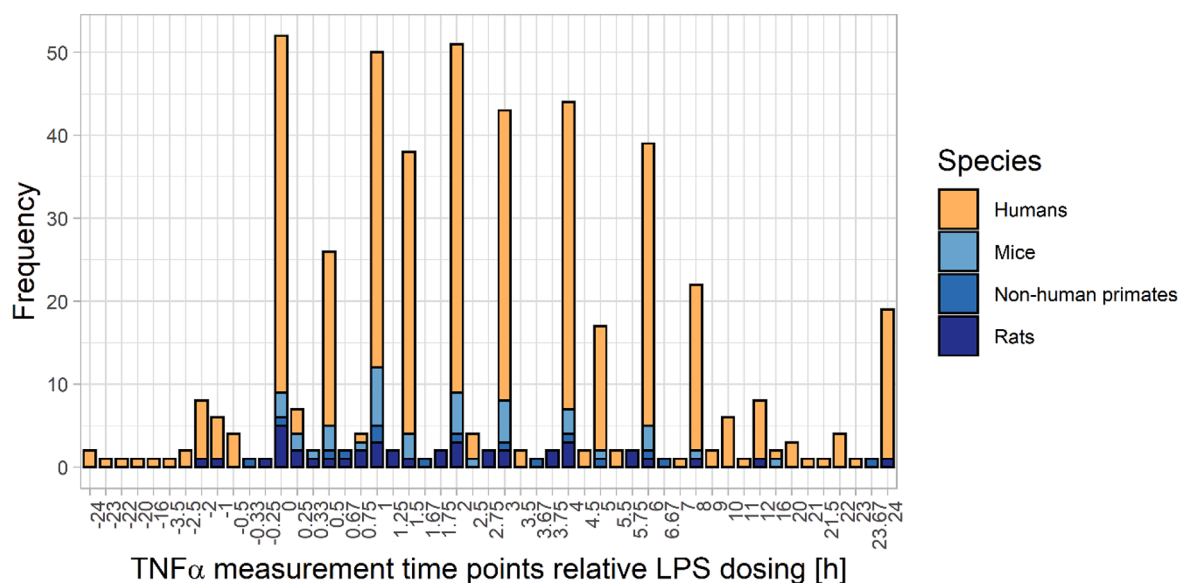
## 4. Discussion

### 4.1. Optimized time delay between LPS and drug administration

The analysis of the median model predicted TNF $\alpha$  response suggest that the orally administered Test Compound A and LPS should be taken simultaneously, such that the inhibiting effect of Test Compound A coincides with the LPS stimulated production of TNF $\alpha$  (Fig. 2 and Table 2). These results question the conventional rule of thumb that LPS should be administered when the drug or drug metabolite reaches its maximum concentration and instead suggest a shorter time delay between drug and LPS administration, depending on the pharmacokinetics of the drug. Specifically, the suggested updated rule of thumb derived from this work would therefore be to administrate the drug such that the time at maximal drug concentration coincides with the maximal stimulated production of TNF $\alpha$ , which approximately occurs 1–1.5 h after LPS administration (Fig. 2). This is reasonable from a biological perspective, since maximum target engagement should be established close in time to the LPS driven TNF $\alpha$  release to achieve an inhibiting effect (Almquist



**Fig. 4.** Visualization of the initial study design (a) in comparison with the optimized study design (b).



**Fig. 5.** Bar chart showing the most commonly used sampling time points for retrieving TNF $\alpha$  response data in the literature, divided by the species used in the particular study. Notice that the distance between bars does not correspond to the actual time difference. For a complete list, see Supplementary Material 1.

**Table 4**

Comparison of points to consider between studies made in humans and animals (data taken from the literature study, see Supplementary 2).

Points to consider	Humans - Median (Mode)	Animals - Median (Mode)
Number of measurements per individual	9 (6)	7.5 (8)
Total number of subjects	16 (16)	19 (12)
Number of dosing regimens (including control)	2 (2)	2.5 (3)
Time course duration [h]	23.75 (24)	5.71 (6)
Minimum time difference between measurements [h]	0.5 (0.5)	0.5 (0.25)
Time delay between LPS administration and first measurement [h]	0 (0)	0 (0)
Time delay between drug and LPS administration (relative LPS administration) [h]	-2 (-2)	-0.25 (-0.25)
$t_{max}$ for TNF $\alpha$ response [h]	1.5 (1.5)	1.125 (1)
First non-zero TNF $\alpha$ response [h]	1 (1)	0.71 (1)
Last non-zero TNF $\alpha$ response [h]	6 (6)	4.5 (4)

et al., 2020). Furthermore, administering the drug closer to LPS dosing would give more measurements where both drug and TNF $\alpha$  response is present which then would yield a more informative dose-response relationship (Gabrielsson and Weiner, 2001), at least for a small molecule such as Test Compound A.

While the analysis of the median model predicted TNF $\alpha$  response showed an evident improvement in TNF $\alpha$  suppression for simultaneous Test Compound A and LPS administration (Fig. 2 and Table 2), the TNF $\alpha$  suppression was not as apparent when adding the observation error and inter-individual variability to the model simulations (Fig. 3). Thus, there is no guarantee that an evident TNF $\alpha$  suppression, as an effect of choosing an optimal time delay, will be seen in TNF $\alpha$  response data retrieved from biological experiments. Only when data was simulated from 60 to 90 test subjects a significant difference between some of the groups could be demonstrated, implying that the TNF $\alpha$  suppression as a result of optimal time delay gets overshadowed by the variability in response. This is especially seen for the lowest dose group (0.3 mg/kg) where the inhibiting effect of Test Compound A is not strong enough to provide a visible TNF $\alpha$  suppression. This is however reasonable, since the maximal concentration of Test Compound A for this dose was below its  $IC_{50}$  value (Larsson et al., 2021), and that TNF $\alpha$  release is notoriously

known for showing very high variability (Gozzi et al., 1999; Wang et al., 2007), even when using the same LPS batch and inbred animals as done in this paper. To reduce the effect of variability in TNF $\alpha$ , we recommend to use the same batch of LPS and an inbred animal strain when conducting several studies, as done in Larsson et al. (2021).

Apart from the variability in response, the dynamics of the drug is also an important factor when evaluating the effect of optimal time delay. This is exemplified by the largest dose (30 mg/kg), where maximum inhibition is reached and kept during the entire time span (Fig. 2), naturally yielding no significant effect since there is no change in dynamics. For drugs with short half-lives, it will be very critical to align the timing between drug and LPS dosing such that maximal drug concentration coincide with the maximum TNF $\alpha$  release. A good example for such a case is apratastat, where no inhibition of LPS-stimulated TNF $\alpha$  response was observed after the administration of apratastat 3 h before LPS injection, but maximal inhibition of LPS-induced TNF $\alpha$  release could be seen when the drug was given 0.5 h before LPS (Shu et al., 2011). Even though the underlying mechanisms for the different outcomes are not completely clear, these data clearly indicate that the timing between drug and LPS dosing can be very critical. Therefore, we would recommend to conduct an initial small pilot study and use that information to aim at an informed optimization of the time difference between drug and LPS dosing by modeling. By using this approach, the amount of information with fewer animals have been maximized, unnecessary repetition of experiments has been avoided, and the animals do not need to endure unnecessarily large TNF $\alpha$  responses, thus fulfilling the three Rs (Reduction, Replacement, and Refinement) (Russell and Burch, 1959).

As a last remark, it can be shown analytically that the theory holds for intravenous bolus drug doses and constant infusion rates as well, highlighting that these findings can potentially be put into an even larger context (Supplementary Material 1).

#### 4.2. Optimal LPS dose and sampling time points

The LPS dose should be increased and changes to the sampling time points in the elimination phase of TNF $\alpha$  response should be made, in accordance with the results from the optimization in PopED (Fig. 4 and Table 3). The optimal measurements are well distributed between the absorption, distribution, and elimination phase which correspond well to the general advice of how to plan your experiments (Gabrielsson and

**Table 5**

General points to consider when planning LPS challenge studies. Abbreviations: Intraperitoneal (IP), intravenous (IV), *per oral* (PO), pharmacokinetics (PK), Supplementary Material 2 (S.M. 2). The cases where the suggestions are similar for modeling and experiments, the table element is noted with a (-).

Points to consider	Suggestions, modeling	Suggestions, experiments	Source
Species	Larger animals (rats) allow for full individual profiles. Smaller animals such as mice might require composite profiles, which increases variability	<i>In-vitro</i> whole blood experiments might suggest sensitive species.	S.M. 2
Route of LPS administration	IV can be modelled with fewer parameters compared to extra-vascular administration routes.	IP is a convenient experimental preclinical administration route. Consider planned clinical indication (e.g. inhalation for respiratory disease)	Hamesch et al. (2015)
Route of drug administration	Preferably administration routes with simple PK and inhibiting effect, such as a bolus IV or PO dose, due to the complexity of the TNF $\alpha$ response	Consider intended route of drug administration in clinic (IV or PO)	S.M. 2, Gabrielsson and Weiner (2001)
Drug dosing	Single dosing using multiple dose strengths, that efficiently separates biomarker response	Single dose studies preferred over repeated dosing.	Gabrielsson and Weiner (2001)
Blood sampling: Single time point or time-series measurements	Single time point not descriptive enough and time-series measurements are preferred	-	Gabrielsson and Weiner (2001)
Time difference between LPS and drug administration	Such that maximal drug concentration occurs approximately within the first 30–60 min after LPS administration	Drug and LPS may be given simultaneously for more convenient study protocols, preferably if a large drug concentration is reached under the first 30–60 min.	Fig. 2
Time difference between LPS and first TNF $\alpha$ measurement	0 min, for verifying zero baseline	-	S.M. 2, Figs. 4 and 5
Duration of TNF $\alpha$ measuring	6–8 h to capture the TNF $\alpha$ response, >8 h if required for determining PK profile	Same as for the modeling suggestions, but an even longer duration would be required to ensure a return to baseline	S.M. 2, van Lier et al. (2019)
Number and spacing of sampling points for TNF $\alpha$ response	6–8 sufficient, minimum 0.25 h difference	Note animal welfare guidelines for number of blood samples and maximal blood volumes.	S.M. 2, Diehl et al. (2001), Charan and Kantharia (2013)
Best combination of PK and TNF $\alpha$ response measurements	Try to choose timepoints that are useful for both PK and TNF $\alpha$ .	-	Diehl et al. (2001), Charan and Kantharia (2013)

**Table 5 (continued)**

Points to consider	Suggestions, modeling	Suggestions, experiments	Source
Plasma volume drawn at each time point	Sufficiently large to reduce the variability due to pipetting errors	Depends on the bioanalytical requirements and number of analytes/biomarkers. Note animal welfare guidelines for maximal blood volumes.	Diehl et al. (2001)
Full profile or composite profile	Full profile for every test subject except mice	-	S.M. 2
Pre-dose and post-dose samples required to establish the TNF $\alpha$ baseline	Yes	-	S.M. 2, Fig. 5

Weiner, 2001). Specifically, the measurements are placed such that the TNF $\alpha$  response is well characterized, for example capturing the elimination phase described as a bi-phasic decline (Gabrielsson et al., 2015; Larsson et al., 2021), which is crucial when making an appropriate assessment of the pharmacodynamic properties of the drug (Gabrielsson and Weiner, 2001; Gabrielsson et al., 2015). Moreover, to have two measurements close to the maximum TNF $\alpha$  response is beneficial and informative in the event of time-shifted maximum between TNF $\alpha$  response in presence or absence of drug (Gabrielsson and Weiner, 2001). One problem however is that two measurements are suggested to be taken at the same time point (at 4.25 h, Table 3), which is due to an assumption made in the optimization that the errors in observations at the same time are independent from each other (Nyberg et al., 2012b). This is of course infeasible when conducting the experiments in real life, and since both measurements still carries information, removing one of the samples is not an option either. Instead, we recommend taking one of these measurements and placing them at the end of the study, for example at 5 h or 6 h. For future studies a serial correlation model could be considered, in order to avoid the clustering of sampling time points (Nyberg et al., 2012b).

Although the results from PopED imply that the LPS dose should be increased, this recommendation should be taken cautiously. It is reasonable that the parameter residual standard error would decrease if choosing a large LPS dose close to saturation, since many of the parameters handles this saturated intensity of TNF $\alpha$  response typically seen for increasing LPS doses, for example the maximal stimulatory effect  $S_{max}$ . However, we recognize that the proposed LPS dose of 260  $\mu\text{g}/\text{kg}$  is close to the upper limit (300  $\mu\text{g}/\text{kg}$ ) and chose to analyze the result in further detail before proposing this optimal LPS dose, as the lower error at high LPS doses has to be balanced against potentially better penetrance of drug inhibition and better tolerability at lower LPS doses. When analyzing exactly how a change in LPS dose affects the result, in terms of increase or decrease in objective function value, no visible trend was seen between the two. Moreover, when estimating the model parameters in *NLMEModeling* using simulated data with either the original or optimized LPS dose, no difference in accuracy was noticed. Therefore, although PopED proposes an optimal LPS dose of 260  $\mu\text{g}/\text{kg}$  it is not good enough evidence to encourage using larger LPS doses. Especially since a too large LPS dose could potentially out-compete a small inhibiting effect from a drug. This is especially important since it has previously been noted that there exists an apparent inter-occasion variability in LPS challenge models affecting the maximal TNF $\alpha$  response (Larsson et al., 2021), where the maximal response in this particular study is reached for a LPS dose far below 260  $\mu\text{g}/\text{kg}$ . This explains why the difference in maximal response is relatively small between the original and optimized design (Fig. 4), but due to this

inter-occasion variability the TNF $\alpha$  response could be much larger in other studies. Regardless of exactly which LPS dose is the optimal, model simulations for the median individual showed that for the largest drug dose, where 80% inhibition is kept over the duration of the experiment, the TNF $\alpha$  response was measurable (above the lower limit of quantification  $\hat{\alpha}$  0.0125  $\mu\text{g/L}$ ) for all measurements for LPS doses above 3  $\mu\text{g/kg}$ . Thus, to be certain that the drug effect is not too effective such that TNF $\alpha$  response becomes immeasurable, we recommend using LPS doses above 3  $\mu\text{g/kg}$ , but it is difficult to know how well an LPS dose of 260 would perform over a dose of 30, for example. Another option would be to use more than one LPS dose, as proposed in [Gabrielsson et al. \(2015\)](#), to further investigate the properties of the challenger.

As a last remark, the results from the analysis of optimal study design variables, including the time delay between drug and LPS administration, naturally depend on the pharmacokinetic and pharmacodynamic properties of the studied drug, the model generating the simulated data, as well as the choice of optimal design tool and corresponding settings. In this case the non-linear mixed effects model describing TNF $\alpha$  response proposed in [Larsson et al. \(2021\)](#), both in presence and absence of Test Compound A, has been used as a proof-of-concept together with the PopED settings described in [Section 2.3.2](#). The described principles for the optimization of the study design should hold true for all drugs and all routes of administration, but the specific outcomes can naturally be different for other drugs. Nevertheless, we are confident that Test Compound A serves as a good example for a typical drug with good drug-like properties and nanomolar potency. Thus, the proposed model serves as a good representation of common TNF $\alpha$  response data, and appropriate settings in the optimization. For further discussion concerning the model assumptions and alternative PopED settings, see Supplementary Material 1.

#### 4.3. General guidelines for constructing LPS challenge studies and translation to other test subjects

Many similarities can be found when comparing LPS challenge models in animals and humans, suggesting that findings when optimizing preclinical study design can be translated to clinical studies ([Fig. 5](#) and [Table 4](#)). In particular, the TNF $\alpha$  response has a very similar behavior in humans compared to animals with an initial time delay of 30 min, a peak response around 1.5 h, and a return to baseline after 6–8 h (see [Table 4](#), Supplementary Material 1 and 2, and [van Lier \(2019\)](#)). Although the LPS dose is very different, ranging approximately from 0.2 to 4 ng/kg for intravenous doses in humans and 3 to 300  $\mu\text{g/kg}$  in animals, this pattern is still observed. Despite the large difference in LPS dose the time course of TNF $\alpha$  response are relatively similar, suggesting that the optimized sampling time points could be beneficial in humans as well. Therefore, conducting clinical trials using an optimal study design derived from a preclinical study would be an interesting continuation of this work, which can be used as verification of the theory.

Although comparisons and recommendations can be made, as done here, the one thing that should always be kept in mind is that to always keep the purpose of the study in sight. This can be seen in [Table 4](#), especially in the differences in number of dosing regimens and time course duration. Since on one hand, the goal in a preclinical study is usually to determine the pharmacodynamic effect of the drug of consideration. On the other hand, an LPS challenge in a clinical trial rather serves as a proof-of-concept to determine if the drug has an effect in humans with regards to biomarker modulation, and what the appropriate dosing regimen could be in future clinical trials. Therefore, multiple drug doses are of less importance while it is more important to follow the subject for a longer time period, hence the larger time course duration. The summaries in [Tables 4](#) and [5](#) serve as a good starting point for general recommendations and to refine the details one needs to find more specific examples that better resembles your purpose, where a list of examples is provided in Supplementary Material 2.

## 5. Conclusions

This work addresses the many challenges in LPS challenge studies and analyzes some of the most central study design variables in further detail, using model simulations and optimal design. We propose a new rule of thumb when choosing the time delay between drug and LPS administration, such that the time at maximal drug concentration coincides with the maximal stimulated production of TNF $\alpha$ , for maximizing the apparent drug effect on TNF $\alpha$  response. Furthermore, the optimization of the LPS dose and sampling time points using PopED showed that the placement of measurements after maximal TNF $\alpha$  response are crucial when optimizing the study design, and that a LPS dose over 3  $\mu\text{g/kg}$  should be sufficient. Lastly, the chosen case study design and optimization results correspond well with the data found in literature, which validates our results. Furthermore, comparing LPS challenge studies in preclinical and clinical settings from literature suggest that their study designs are similar, implying that results from optimal design in preclinical studies could be used for clinical studies as well. We are convinced that combining the general concepts from this case study with the drug- and system specific information retrieved from an initial small pilot study will create a robust basis for designing future preclinical and clinical studies.

### CRedit authorship contribution statement

**Julia Larsson:** Conceptualization, Methodology, Software, Formal analysis, Investigation, Writing – original draft. **Edmund Hoppe:** Formal analysis, Resources. **Michael Gautrois:** Formal analysis. **Marija Cvijovic:** Conceptualization, Writing – review & editing, Supervision. **Mats Jirstrand:** Conceptualization, Writing – review & editing, Supervision.

### Declaration of Competing Interest

None.

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### Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:[10.1016/j.ejps.2022.106256](https://doi.org/10.1016/j.ejps.2022.106256).

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