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### Article

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1 **A systems toxicology paracetamol overdose framework – accounting for high risk**  
2 **individuals**

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15 **Abstract**

16 The most commonly prescribed painkiller worldwide, paracetamol (acetaminophen, APAP) is  
17 also the predominant cause of acute liver failure (ALF), and therefore paracetamol-induced  
18 liver toxicity remains an important clinical problem. The standard clinical treatment framework  
19 for paracetamol overdose currently allows for antidote therapy decisions to be made based  
20 on a nomogram treatment line. This treatment threshold is lowered for patients adjudged to  
21 be highly susceptible to liver injury due to risk factors such as anorexia nervosa or bulimia.  
22 Additionally, both the original and adjusted clinical frameworks are highly dependent on  
23 knowledge from the patient regarding time since ingestion and initial dose amount, both of  
24 which are often highly unpredictable. We have recently developed a pre-clinical framework for  
25 predicting time since ingestion, initial dose amount and subsequent probability of liver injury  
26 based on novel biomarker concentrations. Here, we use identifiability analysis as a tool to  
27 increase confidence in our model parameter estimates and extend the framework to make  
28 predictions for both healthy and high-risk populations. Through pharmacokinetic-  
29 pharmacodynamic model refinement, we identify thresholds that determine whether necrosis  
30 or apoptosis is the dominant form of cell death, which can be essential for effective ALF  
31 interventions. Using a single blood test, rather than the multiple tests required in the current  
32 clinical frameworks, our model provides overdose identification information applicable for  
33 healthy and high-risk individuals as well as quantitative measures of estimated liver injury  
34 probability.

35 **Keywords**

36 APAP; DILI; biomarkers; *in-silico*; pharmacokinetics; identifiability.

## 37 1 Introduction

38 Paracetamol (acetaminophen, APAP), the most commonly prescribed painkiller in the world  
39 [1], is also the leading cause of acute liver failure (ALF) [2] and therefore represents a  
40 concerning global health issue [3]. In England and Wales, APAP poisoning results in  
41 approximately 40,000 hospital admissions, 20 liver transplants and 200 deaths per year [4].  
42 Between 2015-2016, there was an 11% increase in deaths involving APAP in the UK [5]. In  
43 the USA, ALF is responsible for approximately 56,000 emergency room visits, 2,600  
44 hospitalisations and 500 deaths per year [6]. Considering this, the clinical overdose  
45 intervention treatment framework is found to be surprisingly sub-optimal. Administration  
46 decisions regarding the overdose antidote, N-Acetylcysteine (NAC), are currently based upon  
47 the nomogram treatment line [7] which, though influenced by a measurement of alanine  
48 aminotransferase (ALT), is also heavily dependent on patients' knowledge of initial dose  
49 amount and time elapsed since ingestion; information which is often unreliable. Given this  
50 uncertainty, decisions on whether or not to administer the NAC antidote can be imprecise.  
51 Furthermore, unnecessary NAC administration can cause a range of side effects such as  
52 nausea, vomiting and anaphylactoid reaction, thereby exacerbating the problem of ill-informed  
53 treatment [8]. Such inaccurate treatment decisions have led to an estimated cost of £8.3  
54 million per year in the UK since 2012 [9].

55 APAP is predominantly metabolised in the liver via glucuronidation and sulphation pathways,  
56 with a small fraction being oxidised into the toxic metabolite, NAPQI. Detoxification of NAPQI  
57 occurs via conjugation with hepatic stores of glutathione (GSH) [10]. Therefore, although initial  
58 dose and time since ingestion are known to be the most important indicators of overdose  
59 severity level, additional factors affecting an individual's ability to synthesise or maintain  
60 sufficiently high levels of GSH should also be considered [11]. Such factors may include age,  
61 pre-existing liver disease, concurrent use of alcohol and/or other liver-metabolised  
62 medications, genetic predispositions and acuity/chronicity of APAP use [3]. Patients with

63 known high-risk factors are currently measured against an amended nomogram treatment line  
64 when deciding whether or not to administer NAC [12].

65 Crucially, the availability of GSH is known to be heavily dependent on the nutritional level of a  
66 patient. Therefore, malnourished patients, or those suffering from eating disorders (particularly  
67 anorexia or bulimia nervosa) are deemed to be at a particularly high risk of developing liver  
68 injury following overdose of APAP [11]. Eating disorder admissions have risen by  
69 approximately 34% since 2006 in the UK and, with anorexia having the highest mortality rate  
70 of all psychiatric disorders (often linked to suicide) [13], it is essential that quick and accurate  
71 treatment decisions for these high-risk patients can be made so that fatalities can be avoided.  
72 In addition to an increase in those patients with eating disorders, the prescription of  
73 medications combining APAP with opioids is likely to increase the incidence of unintentional  
74 APAP overdose, particularly in the USA where there is currently an opioid addiction epidemic  
75 [14]. Furthermore, the number of alcohol-related hospital admissions in England rose by 22%  
76 in the 10-year period leading up to 2016 [15]. Since patients with opioid and alcohol-related  
77 issues are known to have a higher susceptibility to APAP toxicity, there is an increasingly  
78 urgent need to improve intervention efficacy for the growing number of patients in these high-  
79 risk groups.

80 Some of the most important goals of medicine are to reduce pain and prolong life [16] and  
81 scientific research has allowed for significant advancements in achieving these goals.  
82 However, there is also an increasing impetus to reduce the extent of animal testing required  
83 to conduct medical research [17]. Quantitative systems toxicology (QST) modelling comprises  
84 a useful tool to reduce and refine animal testing and is now considered as both an essential  
85 component of modern toxicity testing and a foundation for individualised therapeutic treatment  
86 [18]. However, the utility of a QST model, as an abstract representation of the true biology, is  
87 limited by its simplifying assumptions and consequently there are often multiple aspects of the  
88 model that contain uncertainty. Not all states of a dynamic model can be directly measured

89 experimentally, and conversely, not all experimental data may be useful for model calibration,  
90 since the data itself may contain errors not accounted for by the model. These limitations can  
91 raise scepticism around QST model predictions and while it would be unrealistic to attempt to  
92 completely eradicate every level of error, it is crucial that any parameter uncertainties should  
93 be assessed, reported and minimised in order for these models to be truly useful in their  
94 predictions [19]. There are many existing and developing techniques to quantify uncertainty,  
95 and the chosen method often depends on the aims of the model. Identifiability analysis can  
96 be employed to determine whether model parameters can be uniquely identified based upon  
97 the structure of the model and data used, and sensitivity analyses can provide quantification  
98 of the dependency of model outputs to perturbations in the model parameters.

99 We have recently developed a framework that uses a single sample, rather than multiple  
100 sample, approach to biomarker quantification in order to predict the probability of liver injury  
101 [20]. This model was optimised against fed mouse data and therefore is limited to applications  
102 relating to individuals with unimpaired clearance capacity. Through the application of  
103 uncertainty quantification techniques, we here identify areas within the original model structure  
104 that require improvement and use this knowledge to make the structure more relevant to the  
105 APAP toxicity clinical environment. There are many other *in-silico* models which focus on  
106 describing and understanding APAP-induced toxicity. Howell et al. [21] combined a large-  
107 scale, mechanistic mathematical model (DILIsym<sup>®</sup>) with *in-vitro* data to compare DILI  
108 responses across species. Whilst their model has potential utility for *in-vitro* to *in-vivo*  
109 extrapolation, parameter identifiability was not assessed within their study. Reith et al. [22]  
110 clarified the role of glucuronidation and sulphation pathways in the hepatic metabolism of  
111 APAP. Ochoa et al. [23] reported a physiologically-based pharmacokinetic (PBPK) modelling  
112 approach to predict APAP and toxic metabolite concentrations, which were then used to  
113 estimate spatiotemporal cell integrity and the elimination rates of various substances.  
114 Zurlinden et al. [24] used a Bayesian inference approach within a PBPK model to estimate

115 initial APAP dose and quantify its uncertainty based on conventional biomarkers. Our model  
116 aims to optimise patient stratification and overdose treatment by incorporating a panel of novel  
117 mechanism-based biomarkers of increased sensitivity [25]. Remien et al. [26] also used  
118 conventional biomarkers to predict initial dose and time since overdose. Our work in [20]  
119 extended this previous work with the inclusion of novel biomarkers HMGB1 and K18. However,  
120 there is currently only one *in-silico* APAP model which takes into consideration individuals that  
121 may have depleted GSH stores. Navid et al. (2013) developed a multi-compartmental PBPK  
122 model of APAP metabolism in order to understand how nutritional deficiencies and certain  
123 lifestyle choices, such as alcohol consumption, affect GSH regeneration [27].

124 PBPK models are increasingly being utilised to account for blood flow between organs, tissue  
125 partitioning, and predicting localised drug concentrations at the site of action [24, 28].  
126 However, these large-scale models, while more physiologically relevant, favour complexity at  
127 the expense of mathematical tractability and subsequently contain an increased number of  
128 parameters that require estimation. The large scale of the models and amount of parameters  
129 to be optimised, often outweighing the amount and quality of data available for such a task,  
130 renders identifiability of model parameters problematic. Since the distribution and metabolism  
131 of APAP is well known, we opt for a smaller scale model which focuses on liver toxicity  
132 biomarkers. The smaller scale model allows for closer scrutiny of the model itself via  
133 techniques such as sensitivity analysis and identifiability analysis, providing mechanistic  
134 insight into structure, parameterisation and interactions within the system.

135 In this study, we extend our original APAP overdose framework [20] to quantify the effects of  
136 various factors that impact upon GSH availability to develop an improved overdose  
137 identification framework. The mathematical model is refined using techniques from uncertainty  
138 analysis to account for mechanistic changes indicative of suppressed GSH capacity and  
139 optimised against additional data to improve its scope and predictive potential. Analysis of this

140 improved model provides predictions of initial dose, time since ingestion and probability of liver  
141 injury for both healthy and high-risk populations.

## 142 **2 Methods**

### 143 **2.1 Experimental data description**

144 The mathematical model is parameterised against multiple experimental datasets. For the  
145 APAP PK element of the model, four datasets from two separate published studies [25, 26]  
146 recording APAP concentration over time in mice following intraperitoneal administration of 50,  
147 150, 500 and 530 mg/kg doses are used in both the original and extended framework. For the  
148 biomarker PD element of the model, in the original framework one experimental dataset is  
149 used [30] which records biomarkers (GSH, ALT, HMGB1, full and fragmented K18) over time  
150 following a 530 mg/kg APAP dose. In the extended framework, this dataset is also used, but  
151 with the addition of two other datasets from two separate studies [20, 27]. The first, [20],  
152 provides dose response data for mouse biomarker concentrations GSH, ALT, HMGB1 and  
153 fragmented K18 at 5 hours following APAP doses [0,150,300,530,750,1000] mg/kg. The  
154 second, [31], provides biomarker concentrations GSH, ALT, HMGB1, full and fragmented K18  
155 at 5 and 24 hours for both fed and fasted mice following a 530mg/kg APAP dose. All datasets  
156 described were used for optimisation of the model parameters. The data consists of a wide  
157 range of dosing scenarios to the extent that we do not extrapolate beyond these calibration  
158 ranges in subsequent model simulations.

### 159 **2.2 Parameter optimisation**

160 A multi-start technique is applied during parameter optimisation in an attempt to find the  
161 globally optimal parameter set following the restructuring of the original model [20]. All  
162 dynamic models are optimised by minimising the sum of squared errors (SSE) between model  
163 simulated output and the experimental data **described in section 2.1** (fminsearch, Matlab [32]).

164 The Matlab minimisation function uses a Nelder-Mead search algorithm to iteratively search  
7



165 the parameter space until a local minimum is found [33]. Latin hypercube sampling was used  
166 to generate 1000 different initial estimates for each parameter (ranges defined in the  
167 supplementary material).

### 168 **2.3 Identifiability analysis**

169 Identifiability analysis is performed to visualise changes in SSE (deviation between model  
170 output and experimental data) following parameter perturbations and, subsequently, to  
171 determine the identifiability of each parameter in the model. We apply a method of identifiability  
172 analysis similar to the profile likelihood approach defined by the FRIAS research group [34].  
173 Parameter estimates are either identifiable (unique minimum), practically unidentifiable  
174 (monotonic response), or structurally unidentifiable (negligible response) [35]. During this  
175 analysis, each individual parameter is tested separately for identifiability. This “test parameter”  
176 is varied by 20% intervals (from -50% to +200% of its original value). In each iteration, the  
177 modified test parameter is fixed, while all the other parameters in the model are varied in two  
178 ways: fixed at the optimum values (as found from previous multi-start optimisation); or  
179 randomly sampled from a Latin hypercube (bounds for sampling can be found in the  
180 supplementary material). For each test parameter iteration, the parameter set corresponding  
181 to the lowest SSE value is then identified. In this analysis, an identifiable parameter is defined  
182 as a parameter which, when perturbed from its initial (optimal) value (both positively and  
183 negatively), results in an increased SSE and therefore predicts a greater error between the  
184 model output and the data. If the SSE increases on only one side (i.e. in the positive or  
185 negative direction) of the test parameter, and remains relatively unchanged on the other side,  
186 this parameter is defined as practically unidentifiable; that is, either increasing or decreasing  
187 the test parameter value causes an increased error between the model output and the data.  
188 However, since the error between model output reduces as we head towards the test  
189 parameter, but then remains relatively unchanged for further perturbations in that direction,  
190 we cannot be confident that the parameter is uniquely optimal, since there are multiple values

191 that provide the same approximate SSE. However, since the optimum path does change in  
 192 one direction, we have confidence that we are capturing some of the structure, and often these  
 193 kind of ‘practical unidentifiabilities’ can be resolved by including more calibration data [34]. If  
 194 the SSE value does not change either side of the original test parameter value, this parameter  
 195 is known as structurally unidentifiable. This means that parameter optimisation via data-fitting  
 196 is relatively insensitive to the choice of this parameter; the parameter cannot be uniquely  
 197 determined and therefore even if removed entirely, values of other parameters could be  
 198 suitably adjusted to compensate for the change in the model structure. During both sensitivity  
 199 and identifiability analyses, model parameters are perturbed and subsequent changes in  
 200 model output are studied. However, parameters are not re-optimised during sensitivity  
 201 analysis. Identifiability analysis seeks to determine whether distinct model parameterisations  
 202 could provide the same model solution.

## 203 **2.4 Model refinement**

### 204 *2.4.1 APAP pharmacokinetic model*

205 Three datasets from three separate published studies [20, 26, 27] were used to parameterise  
 206 the APAP pharmacokinetic (PK) model which is developed as an extension to our previous  
 207 framework [20]. The PK model structure remains unchanged and is summarised below for  
 208 completeness. Note that all the data used was obtained in studies of APAP dosing in mice  
 209 (fed or fasted). Two ordinary differential equations (ODEs) are used to represent changes in  
 210 APAP concentration within two PK compartments (central and peripheral) in the following  
 211 system,

$$\frac{d[C_c]}{dt} = \frac{k_a D_0 e^{-k_a t}}{V_c} + k_{21}[C_p] \frac{V_p}{V_c} - k_{12}[C_c] - k_{el}[C_c], \quad (1)$$

$$\frac{d[C_p]}{dt} = k_{12}[C_c] \frac{V_c}{V_p} - k_{21}[C_p], \quad (2)$$

212 where  $[C_c]$  is the central compartment concentration ( $\mu\text{mol/l}$ ) of APAP;  $[C_p]$  is the peripheral  
213 compartment concentration ( $\mu\text{mol/l}$ ) of APAP; and  $k_a$  represents the absorption rate ( $\text{h}^{-1}$ ) from  
214 where APAP is administered (the peritoneal cavity in this case). The initial dose ( $\mu\text{mol/kg}$ ) is  
215 given by  $D_0$ ;  $k_{21}$  represents the transfer rate ( $\text{h}^{-1}$ ) from peripheral to central compartment;  $k_{12}$   
216 represents the transfer rate ( $\text{h}^{-1}$ ) from central to peripheral compartment;  $V_p$  is the theoretical  
217 volume ( $\text{l/kg}$ ) of the peripheral compartment;  $V_c$  is the theoretical volume ( $\text{l/kg}$ ) of the central  
218 compartment;  $k_{el}$  represents the overall elimination rate (summation of excretion and  
219 metabolism processes) ( $\text{h}^{-1}$ ); and  $t$  represents the time variable (h). Further details of the  
220 APAP pharmacokinetic model can be found in our previous publication [20].

#### 221 2.4.2 Pharmacodynamic models

222 Previously, the pharmacodynamic (PD) element of the model was parameterised using a  
223 dataset consisting of GSH and biomarker (ALT, HMGB1, full and fragmented K18) time-course  
224 concentrations following a 530 mg/kg intraperitoneal APAP dose [20]. In this paper, we extend  
225 this optimisation to also include dose-response data consisting of plasma biomarker  
226 concentrations at 5 hours following APAP doses ranging from 0-1000 mg/kg [20] and a dataset  
227 consisting of biomarker concentrations at 5 and 24 hours for both fed and fasted mice following  
228 a 530 mg/kg APAP dose [31]. This extension is necessary in order to account for differing  
229 mechanisms of cell death, i.e. apoptosis versus necrosis, and also to account for an increased  
230 dosing range more representative of the clinical environment.

##### 231 2.4.2.1 Glutathione dynamics

232 In our model, paracetamol toxicity biomarker dynamics are assumed to be directly dependent  
233 on GSH depletion; i.e., during APAP overdose cases when GSH pools deplete, NAPQI  
234 accumulates potentially leading to liver toxicity and associated biomarker release. The GSH  
235 model component remains identical to our previously defined model [20], namely:

$$\frac{d[gsh]}{dt} = k_o(gsh_0 - [gsh]) - \frac{\xi k_{el} C_c [gsh]}{[gsh] + k_{pr}}, \quad (3)$$

236 where  $k_o$  is the basal removal rate ( $\text{h}^{-1}$ ) of GSH (including background usage);  $gsh_0$  is the  
 237 baseline concentration ( $\mu\text{mol/l}$ ) of GSH in the APAP-free steady state;  $\xi$  is the proportion of  
 238 eliminated APAP that is transformed into NAPQI;  $k_{el}$  is the APAP elimination rate;  $k_{pr}$  is the  
 239 ratio of NAPQI forming other protein adducts, relative to being detoxified by GSH, and  
 240  $[gsh]$  represents the concentration ( $\mu\text{mol/l}$ ) of GSH. Further details on the derivation of the  
 241 GSH model can be found in the supplementary material of our previous study [20]. For the  
 242 fasted case, basal GSH dynamics are modified to simulate a delay in GSH repletion due to  
 243 depleted co-factors stemming from the reduced food intake. These slower dynamics are  
 244 incorporated by rescaling  $k_o$  by an additional parameter,  $\delta < 1$  (so that  $k_o$  becomes  $\delta k_o < k_o$ ).

#### 245 2.4.2.2 ALT and HMGB1 dynamics

246 The toxic response of biomarkers ALT and HMGB1 to APAP overdose is mathematically  
 247 modelled as in the previous study [20], namely:

$$\frac{d[r]}{dt} = r_0 k_{out} \left( \frac{R_{50}^n + gsh_0^n}{R_{50}^n} \right) \left( 1 - \frac{g_{max} [gsh]^n}{R_{50}^n + [gsh]^n} \right) - k_{out} [r], \quad (4)$$

248 Where  $[r]$  is the biomarker concentration ( $\mu\text{mol/l}$ ),  $r_0$  is the respective biomarker baseline  
 249 ( $\mu\text{mol/l}$ );  $k_{out}$  is the natural decay rate ( $\text{h}^{-1}$ ) of the biomarker;  $R_{50}$  represents the concentration  
 250 ( $\mu\text{mol/l}$ ) of (GSH) at which the biomarker response to GSH is half its maximal rate; and  $n$  is a  
 251 Hill coefficient indicating the steepness of the biomarker response [36]. Parameter  $g_{max}$   
 252 represents the maximal regulatory effect of GSH on biomarker production. In the fed case, we  
 253 set  $g_{max} = 1$  such that GSH depletion is solely responsible for augmented biomarker  
 254 production and therefore for the APAP-free steady state, the biomarker is only produced at  
 255 low, basal levels maintaining the background steady state value,  $r_0$ . This element of the model  
 256 is therefore identical to the previous study. However, experimental observations show that

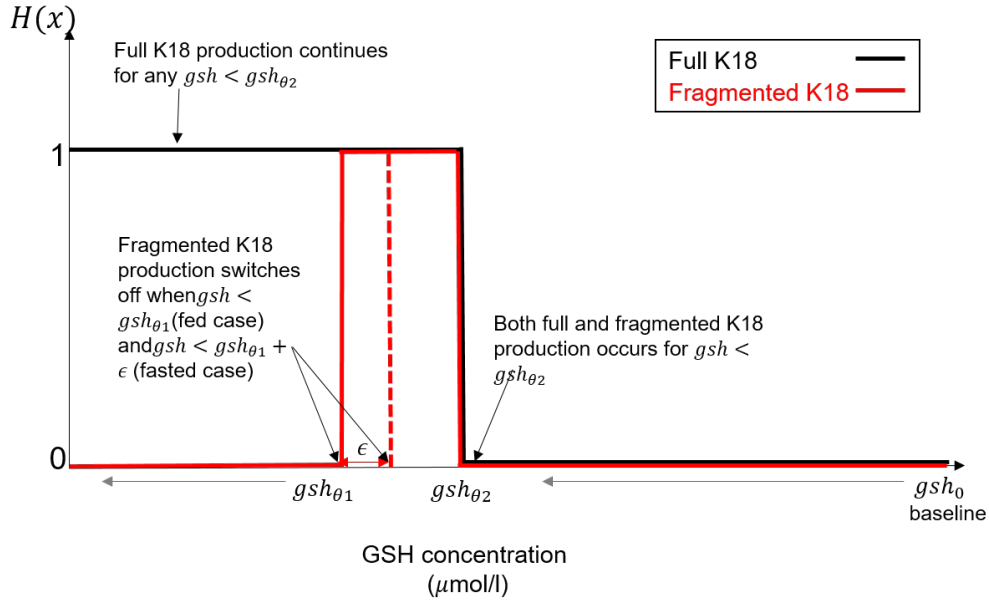
257 background biomarker levels are higher for fasted animals. We therefore take  $g_{max} < 1$  in the  
 258 fasted case to account for these higher background values.

### 259 2.4.2.3 K18 dynamics

260 Keratin-18 (K18) is an intermediate filament protein expressed in abundant levels in  
 261 hepatocytes that undergoes caspase-mediated cleavage during apoptosis, resulting in the  
 262 release of fragmented K18 upon cell death. This feature makes K18 a useful biomarker to  
 263 distinguish between apoptosis and necrosis as the presence of full (as opposed to fragmented)  
 264 K18 instead suggests the occurrence of necrosis [37]. Full and fragmented K18 are therefore  
 265 modelled as necrotic and apoptotic forms of the same single biomarker, K18. However, we  
 266 could not find sufficient data for K18 to properly parameterise a model of the form (4) for both  
 267 full and fragmented K18. We therefore adopt a simple form of K18 using piecewise linear  
 268 representations of (4), as illustrated in Figure 1. That is, the dynamics of the necrotic marker,  
 269 full K18, are modelled in the following way,

$$\frac{d[k18]}{dt} = r_0^{18}k_{out}^{18} + k_{max}k_{in}^{18}H(gsh_{\theta_2} - [gsh]) - k_{out}^{18}[k18], \quad (5)$$

270 where  $r_0^{18}$  is the baseline concentration ( $\mu\text{mol/l}$ ) of full K18;  $k_{out}^{18}$  is the natural decay rate of  
 271 the biomarker;  $k_{in}^{18}$  is the production rate of the biomarker;  $gsh_{\theta_2}$  is the GSH threshold below  
 272 which additional K18 production is induced due to cell death;  $[k18]$  represents the  
 273 concentration of full K18 ( $\mu\text{mol/l}$ ); and  $k_{max}$  is a measure of the production capability of full  
 274 K18 (i.e.,  $0 < k_{max} < 1$ ; since there is a finite quantity of cells, there is a maximum amount of  
 275 biomarker that can be produced). In the fed case, we take  $k_{max} = 1$ . Since fasting in mice  
 276 causes extensive cell loss at early time points [31], the amount of biomarker able to be  
 277 produced from a smaller amount of cells will therefore be smaller. To account for this, in the  
 278 fasted case, we take  $k_{max} < 1$ .  $H(x)$  is the Heaviside function where  $H(x) = 1$  when  $x > 0$   
 279 and  $H(x) = 0$  when  $x < 0$ .



280

281 **Figure 1: Relationship between full and fragmented K18 biomarker production and GSH**  
 282 **concentration.** Black lines represent the full K18 relationship, and red lines represent the  
 283 fragmented K18 relationship (solid lines represent the fed case and dashed lines represent  
 284 the fasted case). As GSH depletes from baseline,  $gsh_0$ , and reaches a certain threshold,  
 285  $gsh_{\theta_2}$ , production of full and fragmented K18 begins. Full K18 production continues for any  
 286 GSH concentration below this threshold; however, fragmented K18 production ceases beyond  
 287 a GSH concentration of  $gsh_{\theta_1}$  in the fed case and  $gsh_{\theta_1} + \epsilon$  in the fasted case.

288 Similarly, the dynamics of fragmented K18 are modelled in the following way,

$$\frac{d[fk18]}{dt} = r_0^{f18} k_{out}^{f18} + k_{in}^{f18} \left( H([gsh] - gsh_{\theta_1} - \epsilon) - H([gsh] - gsh_{\theta_2}) \right) - k_{out}^{f18} [fk18], \quad (6)$$

289 where  $r_0^{f18}$  is the baseline concentration ( $\mu\text{mol/l}$ ) of fragmented K18;  $k_{out}^{f18}$  is the natural decay  
 290 rate ( $\text{h}^{-1}$ ) of the biomarker;  $k_{in}^{f18}$  is the production rate ( $\text{h}^{-1}$ ) of the biomarker;  $gsh_{\theta_2}$  is the GSH  
 291 threshold below which augmented production of fragmented K18 production is initiated,  
 292 and  $gsh_{\theta_1}$  is the GSH threshold below which augmented production ceases due to a switch  
 293 from apoptosis to necrosis.  $[fk18]$  is the concentration ( $\mu\text{mol/l}$ ) of fragmented K18. For the fed  
 294 case, production begins at  $gsh_{\theta_2}$  and ceases at  $gsh_{\theta_1}$ . For the fasted case however, necrosis  
 295 will be more apparent than apoptosis, and less GSH depletion will be required before  
 296 apoptosis is no longer sustainable. To account for this,  $\epsilon$  represents the change in GSH

297 threshold denoting the switch to necrosis, i.e.,  $\varepsilon = 0$  in the fed case and  $\varepsilon > 0$  in the fasted  
298 case.

299 All initial conditions and parameter values for the PKPD model can be found in the  
300 supplementary material.

## 301 **2.5 Statistical analysis**

### 302 *2.5.1 Virtual population simulation*

303 Model predictions are made for three virtually simulated populations: healthy, high-risk, and a  
304 mixture of healthy and high-risk individuals. Healthy populations are based on biomarker  
305 concentrations simulated with parameter values derived from fed data. High-risk populations  
306 are based on biomarker concentrations simulated with parameter values derived from fasted  
307 data. The mixed population is based on a weighting of the biomarker concentrations simulated  
308 with parameter values derived from fed/fasted data, with respect to the proportion of  
309 healthy/high risk patients that are seen in the clinic. Craig et al. [38] analysed overdose  
310 patterns in 663 patients over 16 years and found that 42.3% patients had psychiatric history,  
311 45.3% had alcohol abuse, and 44.7% combined the overdose with alcohol. In line with this,  
312 44.1% of the population is assumed to be high-risk and 55.9% are assumed to be healthy in  
313 our mixed population case.

314 Each virtual population dataset consists of 1000 independent and individually distributed *in-*  
315 *silico* individuals, given a random dose selected from a uniform distribution of range 0-1000  
316 mg/kg. Biomarker concentrations are subsequently extracted at a random time-point from a  
317 uniform range between 0-24 hours. Simulated concentrations are normalised in the range [0,1]  
318 using the min-max normalisation method [39] to account for varying orders of magnitude.  
319 Experimental noise is replicated in the *in-silico* data by applying observed *in-vivo* standard  
320 deviations in biomarker concentrations from an APAP study performed by Antoine et al. (2009)  
321 (ALT s.d = 11.22, HMGB1 s.d = 0.00097, K18 s.d = 2.39, fragmented K18 s.d = 0.12  $\mu\text{mol/l}$ ).

## 322 2.5.2 *Predicting time since administration and initial dose*

### 323 2.5.2.1 *Multiple linear regression*

324 Normality tests indicate that the simulated data is non-normally distributed [40]. Improvements  
325 to the linear model, made by employing interaction/polynomic terms, are insignificant, and  
326 hence a robust multiple linear regression model [41] is applied to the *in-silico*-derived data in  
327 order to predict time since administration and initial dose.

### 328 2.5.2.2 *Visualisation*

329 For each *in-silico* individual, the t-Distributed Stochastic Neighbour Embedding (T-SNE)  
330 method [42] is applied to visualise the dataset constituted by the aforementioned variables  
331 (APAP, GSH, ALT, HMGB1, K18, fragmented K18). Two-dimensional scatter plots of the  
332 embedded data are employed in order to examine class structure and separability whilst  
333 retaining model variation.

### 334 2.5.2.3 *Classification*

335 In order to test the predictive potential of biomarker concentrations, critical ranges for  
336 predicting time since administration are defined as (0-2], (2-5], (5-10], (10-15] and (15-24]  
337 hours. For dose, the ranges are [0-200], [201-400] and [401-1000] mg/kg, capturing  
338 therapeutic, small, and large (overdoses), respectively. A number of classification techniques  
339 are applied and compared, further details of which can be found in the supplementary  
340 information of our previous study [20].

## 341 2.5.3 *Predicting probability of liver injury*

342 Our previously defined binary logistic regression framework [20] uses experimental biomarker  
343 time-course data [30] to predict a corresponding histology score for each mouse. The whole  
344 panel of biomarkers was tested, and analysis found HMGB1 concentration to be the most

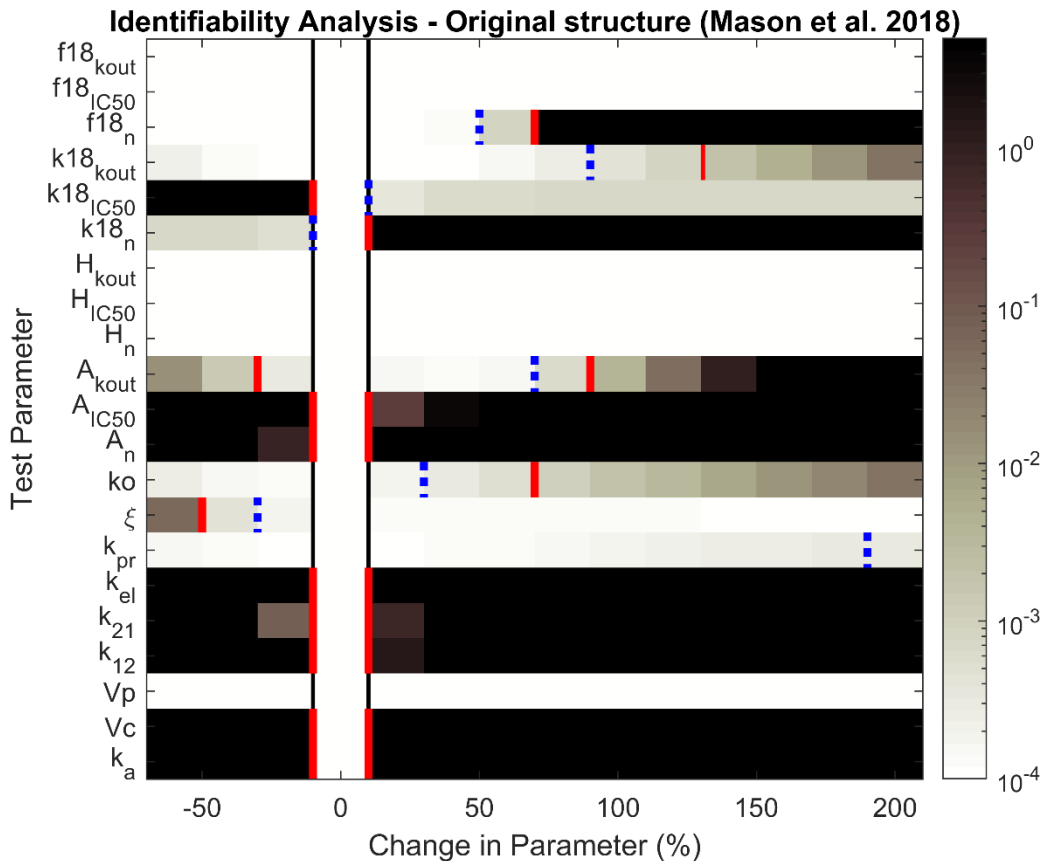


345 significant in predicting the probability of liver injury. The resultant logistic regression model  
346 is used here in combination with PK-PD model simulations to predict the drug-induced liver  
347 injury (DILI) probability [43].

### 348 **3 Results**

#### 349 **3.1 Identifiability analysis – original model structure**

350 Parameters in the original model structure are individually perturbed to visualise resultant  
351 differences between model output and experimental data. These changes are assessed to  
352 determine the identifiability of each parameter and identify where changes may be necessary.  
353 As seen in Figure 2, 10 out of 21 parameters are identifiable. The ALT component of the model  
354 is identifiable, and all but one of the parameters in the PK component are identifiable.  
355 However, the HMGB1 component of the model is structurally unidentifiable, and practical  
356 unidentifiabilities exist in all other elements of the model. Five parameters are identified as  
357 practically unidentifiable. There are 3 within the GSH component: the ratio of NAPQI forming  
358 other protein adducts relative to being detoxified by GSH ( $k_{pr}$ ); the proportion of eliminated  
359 APAP that is transformed into NAPQI ( $\xi$ ); the basal removal rate of GSH ( $k_o$ ). The remaining  
360 practical unidentifiabilities are found within the K18 and fragmented K18 components of the  
361 model: the decay rate of full K18 ( $K18_{kout}$ ); and the fragmented K18 hill coefficient ( $f18_n$ ).  
362 The remaining 6 parameters are structurally unidentifiable. These include the theoretical  
363 volume of the peripheral compartment ( $V_p$ ) from the PK component and all parameters from  
364 the HMGB1 component: the hill coefficient ( $H_n$ ); the GSH concentration at which the  
365 augmented production rate of HMGB1 reaches 50% of its maximum ( $H_{IC50}$ ); and the decay  
366 rate ( $H_{kout}$ ). Two out of three parameters within the fragmented K18 component are  
367 structurally unidentifiable: the GSH concentration at which the augmented production rate of  
368 fragmented K18 reaches 50% of its maximum ( $f18_{IC50}$ ); and the decay rate ( $f18_{kout}$ ).



369

370 **Figure 2: Identifiability analysis of the original model structure** [20]. Each test parameter,  
 371 was fixed at 20% intervals, and the other parameters in the model allowed to vary. The  
 372 percentage change of resultant optimised function values (SSE) are plotted at each interval  
 373 (on log-scale). The lowest SSE change is represented by a white box; darker colours show an  
 374 increase in SSE change. The highest SSE change is represented by a black box. Blue dashed  
 375 bounds indicate where the parameter is identifiable at the 0.5% level. Red bounds indicate  
 376 where the parameter is identifiable at the 1% level. A parameter is identifiable at the 1% level  
 377 if it is bounded by red in both the positive and negative parameter-change directions. If the  
 378 parameter is bounded by red in one direction and blue in the other direction, it is identifiable  
 379 at the 0.5% level but practically unidentifiable at the 1% level. A parameter is practically  
 380 unidentifiable if it is bounded by red/blue in either the positive or negative parameter change  
 381 direction, and unbounded in the opposite direction. A parameter is structurally unidentifiable if  
 382 it is unbounded in both positive and negative parameter-change directions (no red/black  
 383 bounds exist for the whole range of parameter changes). For example,  $A_{IC50}$  is identifiable at  
 384 the 1% level,  $K18_{IC50}$  is identifiable at the 0.5% level but practically unidentifiable at the 1%  
 385 level,  $f18_n$  is practically unidentifiable,  $V_p$  is structurally unidentifiable.

### 386 3.2 Re-parameterisation

387 Following model restructuring and re-parameterisation against the multiple datasets  
 388 (increased dose range and fasted data), we identify a number of parameter adjustments. In

389 cases where parameters are common to both model structures, percentage changes in their  
390 values following re-parameterisation are shown in Table 1. The baseline level of GSH,  $GSH_0$ ,  
391 is now optimised against the data rather than fixed as in the previous model. The resultant  
392 value reduces by almost 20% to 559.47  $\mu\text{mol/l}$ . The proportion of CYP-activated APAP that is  
393 transformed into NAPQI,  $\xi$ , increases to around 80%, but there is also an increased level of  
394 NAPQI detoxification resulting from the re-parameterisation (represented by a 22.1%  
395 decrease in parameter  $k_{pr}$ , the ratio of NAPQI forming other protein adducts relative to  
396 detoxification). The new parameter,  $\delta$ , incorporates an effective delay in GSH repletion in the  
397 fasted case due to depleted co-factors. The optimised value is 0.0483 and considerably  
398 reduces the timescale of GSH dynamics in the fasted case.

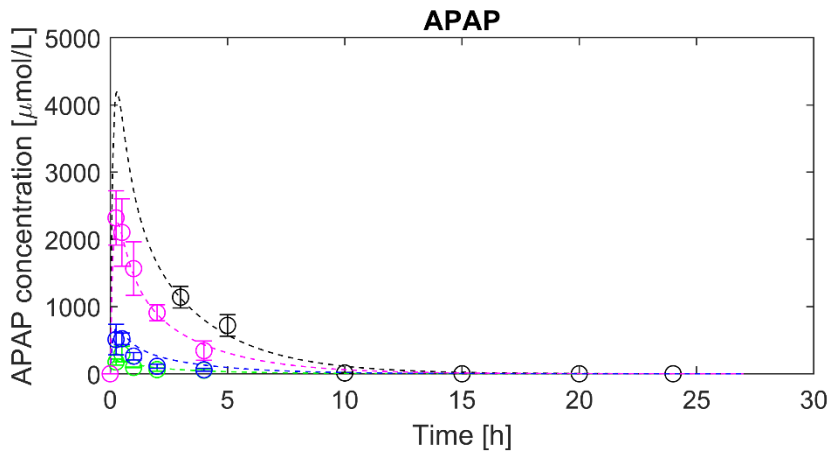
Model	Parameter	Optimised value		
		Original	Re-parameterised	% change
APAP	$k_a$ ( $h^{-1}$ )	9.05	8.6152	-4.8%
	$k_{el}$ ( $h^{-1}$ )	0.52	0.5459	+5%
	$k_{12}$ ( $h^{-1}$ )	0.42	0.4502	+7.2%
	$V_c$ (l/kg)	0.02	0.0220	+10%
	$V_p$ (l/kg)	0.01	0.2102	+2000%
	$k_{21}$ ( $h^{-1}$ )	1.01	1.0315	+2.1%
GSH	$gsh_0$ ( $\mu\text{mol/l}$ )	696.9136	559.47497	-19.7%
	$k_{pr}$	71.06	55.33401	-22.1%
	$\xi$	0.68	0.80571	+18.5%
	$k_o$ ( $h^{-1}$ )	0.25	0.78807	+215%
ALT	$R_0$ ( $\mu\text{mol/l}$ )	0.7626	0.7626	0%
	$n$	9.26	4.3324	-53.2%
	$R_{50}$ ( $\mu\text{mol/l}$ )	227.67	35.6531	-84.3%
	$k_{out}$ ( $h^{-1}$ )	0.0002	0.0004	+1%
HMGB1	$R_0$ ( $\mu\text{mol/l}$ )	0.0005	0.0005	0%
	$n$	4.90	2.3445	-51.2%
	$R_{50}$ ( $\mu\text{mol/l}$ )	399.08	75.2828	-81.1%
	$k_{out}$ ( $h^{-1}$ )	0.35	0.0964	-72.5%
Full K18	$r_0^{18}$ ( $\mu\text{mol/l}$ )	0.0146	0.0088	-39.73%
	$k_{out}^{18}$ ( $h^{-1}$ )	0.0007	0.0031	+342.9%
Fragmented-K18	$r_0^{f18}$ ( $\mu\text{mol/l}$ )	0.0642	0.0977	+52.2%
	$k_{out}^{f18}$ ( $h^{-1}$ )	0.02	0.0031	-84.5%

399 **Table 1: Parameter changes following model refinement.** Any parameter that remained  
400 within the new model structure is defined, with its original value and the re-parameterised  
401 value. Percentage changes are also defined.

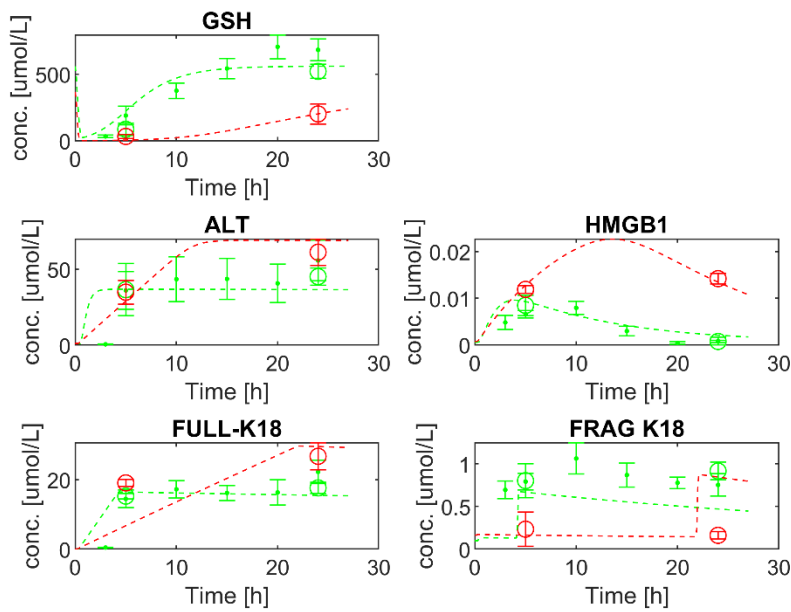
402 Following re-parameterisation, we found that an increased amount of GSH depletion is  
403 required for the GSH-induced ALT response to be half of its maximal value, i.e., 67% GSH

404 depletion was required before whereas almost 94% is required now. Although more GSH  
405 depletion is also required for HMGB1 induction, this biomarker response is still faster than that  
406 of ALT, since it has reached 50% maximal response rate at around 86.5% GSH depletion. For  
407 Full-K18, results from the optimisation suggest that augmented production of the biomarker  
408 will occur when GSH decreases below 174.5205  $\mu\text{mol/l}$  (~68.8% depletion). Fragmented K18  
409 is also induced at this level of GSH; however, if GSH is further depleted to 167.3663  $\mu\text{mol/l}$ ,  
410 augmented production of this apoptosis marker would cease, and the necrotic, full version of  
411 the biomarker would then dominate. In the fasted case, this threshold increases by 5.0457  
412  $\mu\text{mol/l}$  ( $\varepsilon$ ) such that the necrotic switch (as indicated by the absence of K18 fragmentation)  
413 occurs when GSH is depleted beyond 172.412  $\mu\text{mol/l}$ . Optimised model simulations are  
414 plotted and compared with the time-course data (Figure 3) and the dose-response data (Figure  
415 4). Dose-response data was unavailable for full K18 at the time of investigation. Therefore,  
416 the K18 parameters within the model structure are predominantly optimised against high-dose  
417 data which may explain the slight under-prediction shown in the fragmented K18 simulations.

A

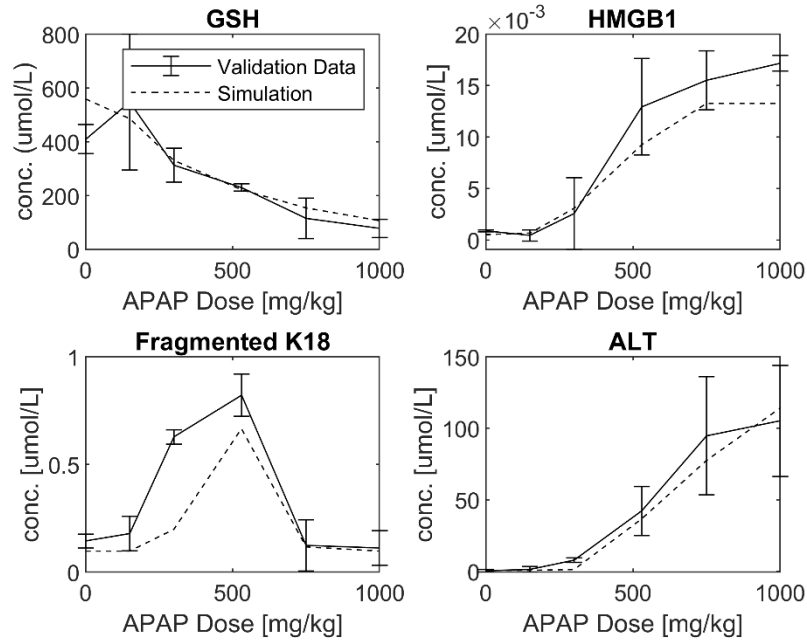


B



418 **Figure 3: Model simulations versus time-course data.** (A): APAP concentration simulations  
419 are plotted for 50 (green), 150 (blue), 500 (pink) and 530 (black) mg/kg doses of APAP. (B):  
420 Fed and fasted simulations (green and red respectively) are plotted for GSH and biomarkers  
421 (ALT, HMGB1, Full and fragmented K18) following a 530 mg/kg dose of APAP.

422



423

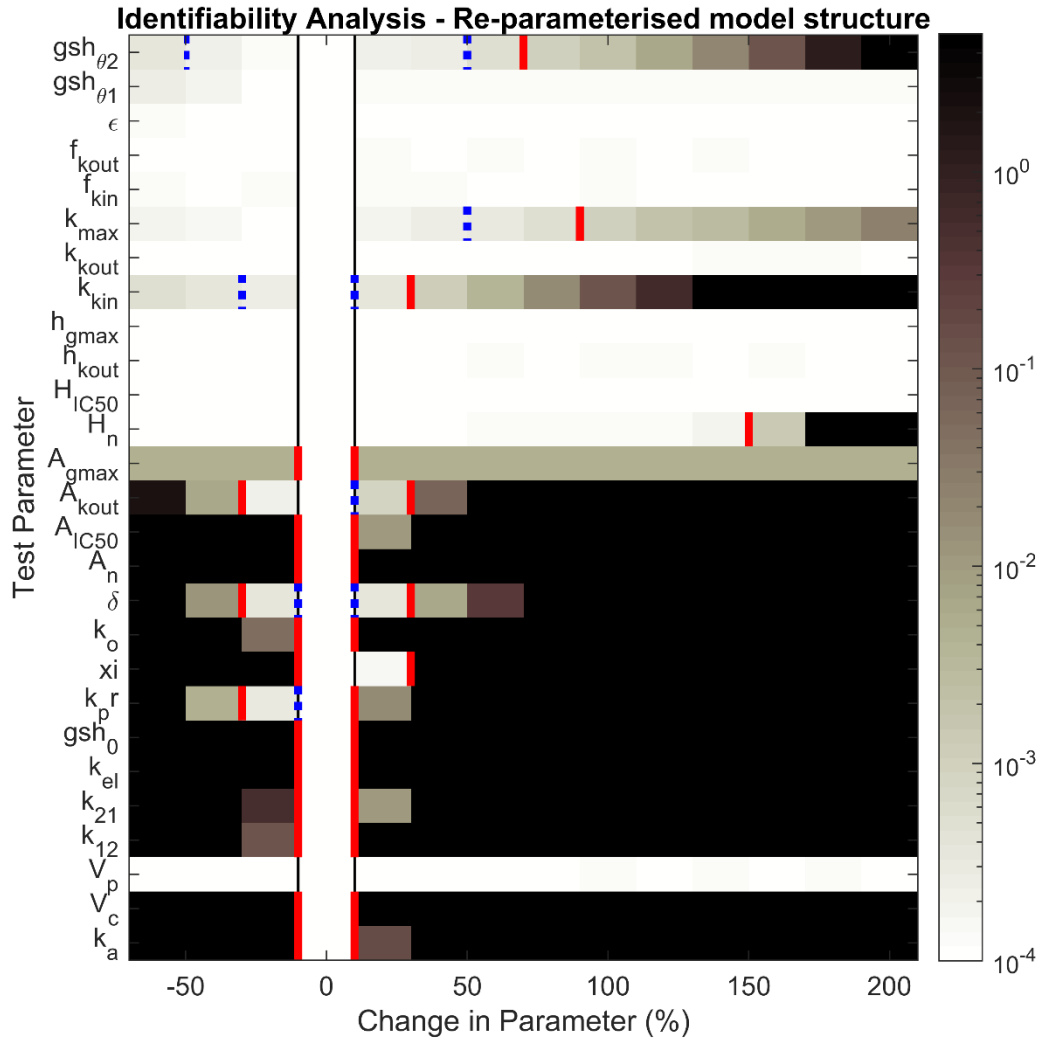
424 **Figure 4: Model simulations versus dose-response data.** Dashed lines represent dose-  
 425 response simulations of GSH and biomarkers (ALT, HMGB1 and fragmented K18) in fed mice  
 426 for a range of APAP doses (0,150,300,530,750,1000 mg/kg). Solid lines represent the  
 427 experimental data each model was calibrated against.

428

### 429 3.3 Identifiability analysis – re-parameterised model

430 Parameters in the refined model are individually perturbed to visualise resultant differences  
 431 between model output and experimental data. These changes are assessed to determine the  
 432 identifiability of each parameter. 16 out of 27 parameters are now identifiable. Parameter  
 433 identifiability has remained for the ALT component, and the GSH component of the model is  
 434 now also completely identifiable. The PK component remains identifiable, other than the  
 435 volume of the peripheral compartment ( $V_p$ ), which remains structurally unidentifiable. There  
 436 are some unidentifiabilities still present within this updated model. Other structurally  
 437 unidentifiable parameters include:  $H_{IC50}$ ,  $H_{kout}$  and  $H_{gmax}$  from the HMGB1 component; and  
 438  $K18_{kout}$ ,  $f_{kin}$ ,  $f_{kout}$ ,  $gsh_{\theta_1}$  and  $\varepsilon$  from the full and fragmented K18 component. Practical

439 unidentifiabilities remain for two parameters: the HMGB1 hill coefficient ( $H_n$ ) and the  
 440 production capability of full K18 ( $k_{max}$ ).



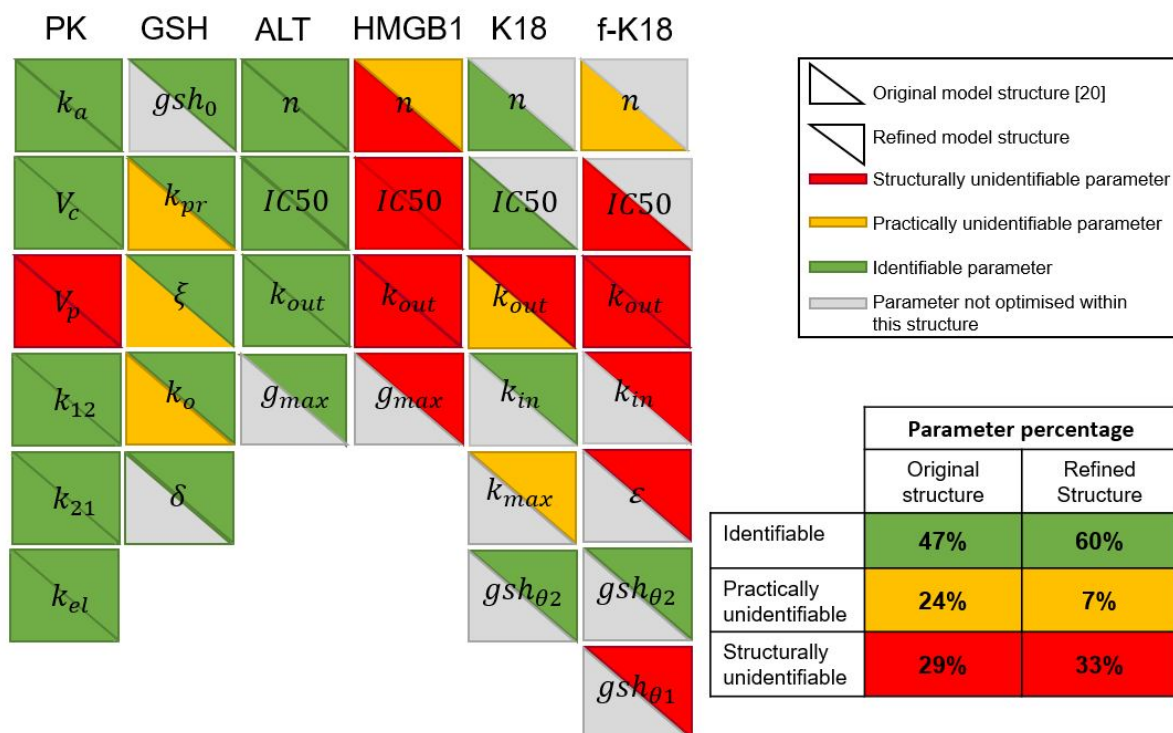
441

442 **Figure 5: Identifiability analysis of the re-parameterised model structure.** Figure  
 443 annotation is the same as for Figure 2. Example results from figure:  $A_{IC50}$  is identifiable at the  
 444 1% level,  $H_n$  is practically unidentifiable,  $V_p$  is structurally unidentifiable.

445 Less than half of the parameters (47%) are identifiable in the original model structure (Figure  
 446 6). Only the ALT component of the model is completely identifiable. 24% of the model  
 447 parameters are practically unidentifiable and 29% are structurally unidentifiable. However,  
 448 following model refinement, the identifiability results have improved; the percentage of  
 449 identifiable parameters has increased to above half of the parameters (60%). Parameter  
 23



450 identifiability has been maintained for the ALT component of the model and the percentage of  
 451 practical unidentifiabilities has reduced from 24% to 7%. The percentage of structural  
 452 unidentifiabilities is approximately the same (29% compared to 33%). Whilst the GSH  
 453 component is unidentifiable in the original model, it is now completely identifiable in the refined  
 454 model. Although unidentifiabilities have improved for parameters in the HMGB1, K18 and f-  
 455 K18 components, there are still some that remain.



456

457 **Figure 6: Parameter identifiability comparison between the original and refined model.**

458 Each column represents a different sub-component of the APAP PKPD model. Each element  
 459 (or square) represents the parameter's identifiability within the model. Triangles in the bottom  
 460 left-hand corner of each element represent the parameter's identifiability in the original model  
 461 structure. Triangles in the top right-hand corner of each element represent the parameter's  
 462 identifiability in the refined model structure. If the triangle is red, the parameter is structurally  
 463 unidentifiable; if the triangle is yellow, the parameter is practically unidentifiable; if the  
 464 triangle is green, the parameter is identifiable. If the triangle is grey, the parameter was not present  
 465 within the corresponding model structure, and therefore could not be tested for identifiability.

466

467

468 **3.4 Predicting an exact time since administration of initial APAP dose**

469 Simulations of the refined model create *in-silico* derived populations. Robust multiple linear  
 470 regression is applied to biomarker concentrations from each population in order to predict an  
 471 exact time since ingestion and initial APAP dose. For the healthy population, an exact time  
 472 since administration can be estimated with an error of approximately 3.9 hours, and an exact  
 473 initial dose amount can be estimated with an error of approximately 66.14 mg/kg (Table 2).

Predictors	Healthy Population		High-risk Population		Mixed Population	
	Time	Dose	Time	Dose	Time	Dose
APAP Conc.	-16.549*** (1.1785)	673.985*** (17.0745)	-9.5093*** (0.5963)	1295.366*** (47.8525)	-18.122*** (0.9542)	909.341*** (17.8005)
ALT conc.	8.972*** (0.9533)	515.625*** (13.8110)	5.5034*** (0.6537)	695.2118*** (52.4551)	1.4573 (0.9228)	655.0837*** (17.2145)
HMGB1 conc.	-22.035*** (0.9113)	-23.3880 (13.2028)	-8.8119*** (0.5440)	367.0774*** (43.6578)	-17.869*** (0.7705)	289.2333*** (14.3736)
FullK18 conc.	7.521*** (1.0632)	479.137*** (15.4035)	21.0427*** (0.4743)	-154.2403*** (38.0652)	22.3833*** (0.9145)	229.0486*** (17.0605)
Fragmented K18 conc.	-4.599* (1.3895)	151.975*** (20.1309)	3.8472*** (0.5962)	86.7133 (47.8445)	-0.1079 (0.8321)	33.8383* (15.5231)
Constant	14.918*** (0.3074)	77.247*** (4.4529)	5.8083*** (0.2527)	-80.145*** (20.2759)	12.6308*** (0.3694)	-65.6354*** (6.8914)
Residual Std. Error (df == 994)	3.907	66.14	2.118	184.1	3.485	73.73

Note:  
 \*p<0.05;  
 \*\*p<0.01;  
 \*\*\*p<0

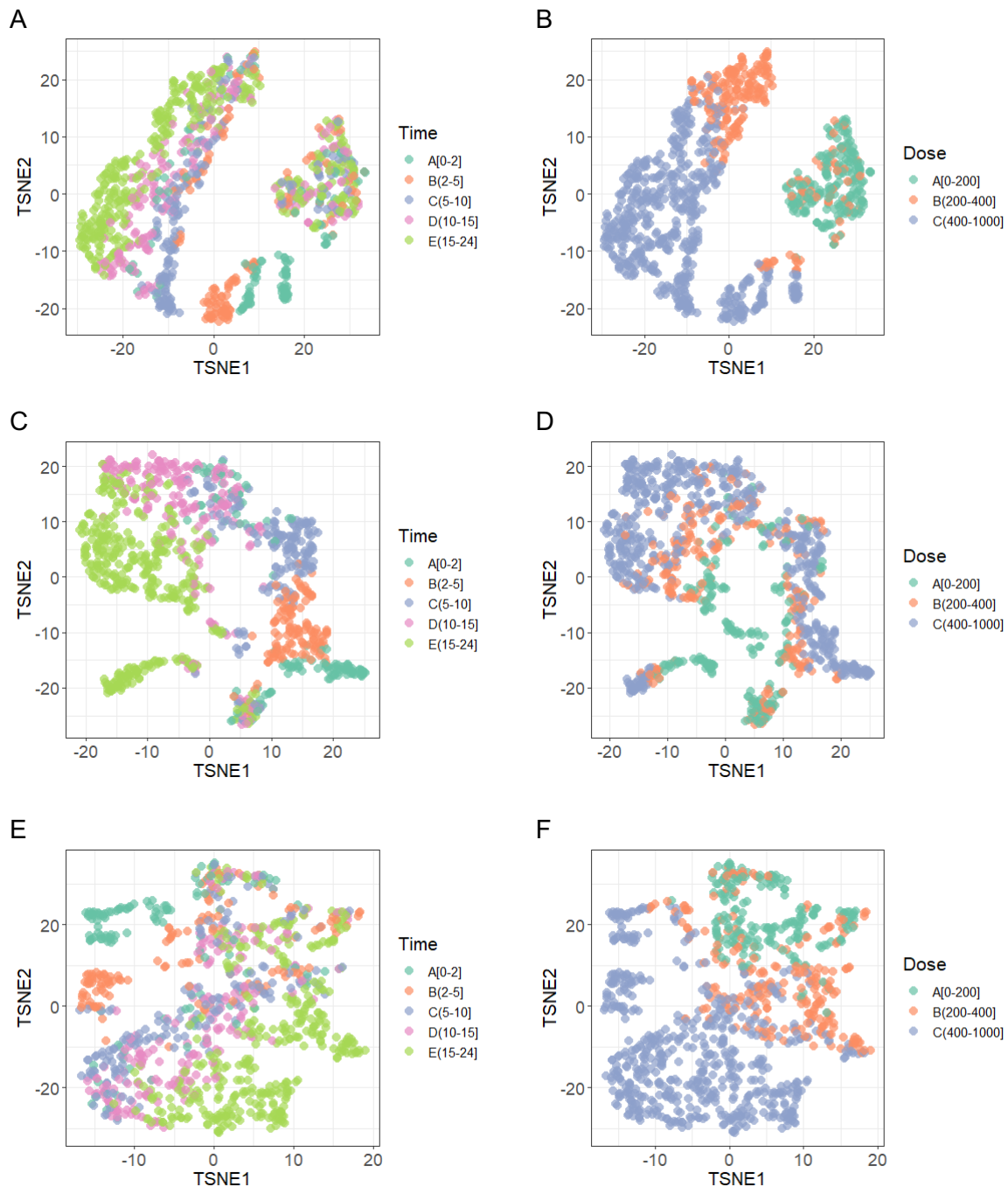
474 **Table 2: Robust multiple linear regression analysis results.** Independent variable  
 475 coefficients for predicting time and dose respectively for healthy, high-risk and mixed  
 476 populations. The first number in each element of the table represents the biomarker coefficient  
 477 in the regression model and the second number represents the corresponding error. For  
 478 example, -16.549 is the APAP concentration coefficient in the healthy population model  
 479 predicting time since administration, and this coefficient has an error of 1.1785. The standard  
 480 error of overall model predictions is provided. The significance of each biomarker in the model  
 481 is indicated by the number of asterisks (see note).

482 Time since administration can be predicted more accurately in the high-risk population, with a  
483 standard error of 2.118 hours. It is much harder to predict the initial dose in the high-risk  
484 population, however, with the standard error being almost triple that of the model for the  
485 healthy population (184.1 mg/kg). Predictions for an assumed mixed population are similar to  
486 that of the healthy population, with a slight improvement in predicting time since administration  
487 (standard error 3.485 hours) and a slight reduction in accuracy for predicting initial dose  
488 (standard error 73.73 mg/kg). All biomarkers are significant in predicting time since  
489 administration in a healthy population and high-risk population independently. However, when  
490 assuming a mixed population, ALT and fragmented K18 are no longer significant. When  
491 predicting dose in a mixed population, all biomarkers are significant; however, HMGB1 is not  
492 significant in the healthy population, and fragmented K18 is not significant in the high-risk  
493 population.

### 494 **3.5 Identifying time/dose category following APAP dose**

495 T-SNE visualisation is applied to the *in-silico* derived data to investigate time/dose class  
496 structure and separability and subsequently various classification techniques are also  
497 employed, using the biomarker concentrations of the *in-silico* observations in an attempt to  
498 classify a time/dose category. Differences in levels of discrimination regarding initial dose and  
499 time since administration for healthy, high-risk and mixed populations can be seen for each  
500 case by embedding the *in-silico* derived data in 2-dimensions using T-SNE (Figure 7). Both  
501 variables, time since administration and initial dose, can be reasonably separated in all  
502 healthy, high-risk and mixed populations. This result is supported by the reasonable accuracy  
503 rates of the classification techniques. Visualising the healthy population (Figure 7A), shows  
504 that there is a cluster of observations on the right-hand-side where the time since  
505 administration is difficult to discriminate from the biomarker concentrations. Visualising this  
506 same cluster of observations with regards to the initial dose, however, shows that these  
507 correspond to low-dose situations (Figure 7B). From measuring biomarker concentrations, a

508 correct time since administration category could be predicted with 69.9% accuracy for the  
509 healthy population, and a correct dose category can be predicted with 91.5% accuracy. These  
510 results are indicated in Table 3. A time since administration category is easier to predict in the  
511 high-risk category (80.4% accuracy). However, predictions for initial dose are less accurate  
512 than the healthy population (79%). If an observation is assumed to be taken from a mixed  
513 population, the prediction accuracy is similar to that of a healthy population, with results of  
514 69.94% and 89.5% for time since administration and initial dose respectively. In our previous  
515 study, a correct time category could be predicted with 72.8% accuracy and a correct dose  
516 category could be predicted with 86.5% accuracy [20]. For a healthy population, the time  
517 classification results have slightly worsened. However, the dose classification results have  
518 slightly improved.



519

520 **Figure 7. Visualisation and classification of time-since-administration and dose results**  
 521 **for healthy, high-risk and mixed populations.** For time-since-administration, dark green  
 522 represents class [0-2), orange represents [2-5), blue represents [5-10), pink represents [10-  
 523 15) and pale green represents [15-24) hours. For dose, green represents [0-200], orange  
 524 represents [201-400] and blue represents [401-1000] mg/kg. TSNE visualisations of *in-silico*  
 525 mouse observations with respect to time since administration and dose can be seen in (A)-(B)  
 526 for the healthy population, (C)-(D) for the high-risk population and (E)-(F) for the average  
 527 population.

528

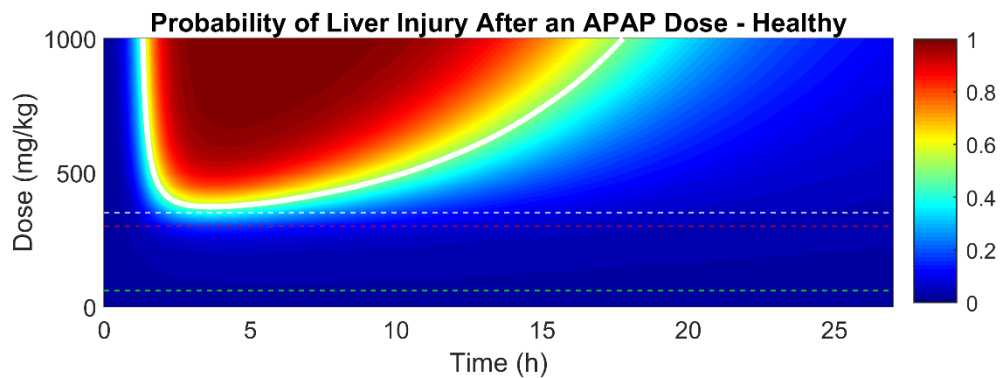
Classification Method	Healthy Population		High-risk Population		Mixed Population	
	Time	Dose	Time	Dose	Time	Dose
KNN	66.2%	91.5%	80.4%	79%	69.5%	87.4%
Regression						
Naïve Bayes	64.4%	91.3%	76.2%	72.7%	68.2%	88.5%
Multinomial logistic regression	68%	90.8%	73.1%	77.1%	75.7%	89.5%
Ordinal logistic regression	53.7%	90%	67.8%	78.1%	57.2%	87.5%
Linear discriminant Analysis	59.3%	90.7%	72.5%	77.9%	65.8%	87.8%
Quadratic discriminant analysis	69.9%	90.4%	76.8%	71.7%	69.94%	86.8%

529 **Table 3: Classification accuracy of predictions based on biomarker concentrations for**  
530 **healthy, high-risk and mixed populations.** For example, the Quadratic discriminant analysis  
531 model can predict the correct time since administration category in the healthy population with  
532 69.9% accuracy.

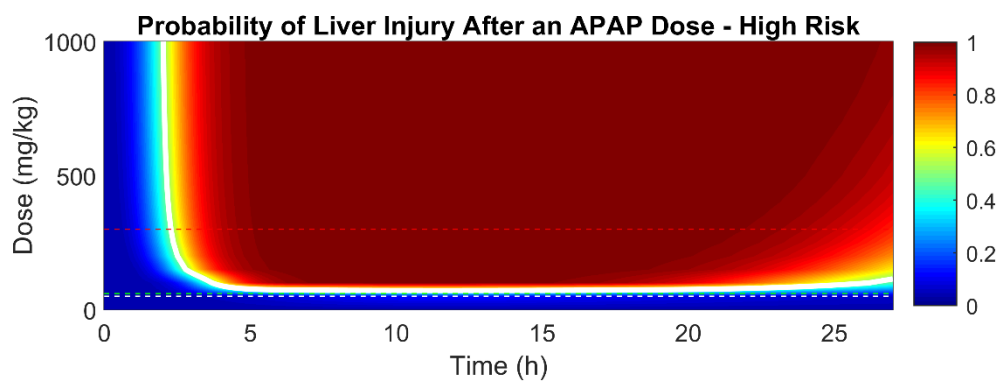
533

534 Figure 8 shows how the probability of liver injury changes over time for both healthy and high-  
535 risk populations, for doses between 0-1000 mg/kg. The model-derived probabilities are  
536 dependent only on HMGB1 concentration (as predicted by our previous logistic regression  
537 model [20]). A threshold probability of 0.5 is used to determine the likelihood of DILI. Any  
538 observation within the white contour boundary is therefore predicted to be representative of  
539 probable liver injury.

A



B



540

541 **Figure 8. Proposed framework for predicting probability of liver injury dependent upon**  
542 **dose, time and HMGB1 concentration.** The plotted frameworks are representative of both  
543 healthy (A) and high-risk (B) populations. In each, the white contour indicates the threshold of  
544 0.5 probability of liver injury; the red dashed-line represents the currently used APAP dose for  
545 toxicity studies in mice; the white dashed-line represents toxic dose as proposed by our model;  
546 and the green dashed-line indicates currently considered therapeutic dose for mice. In the  
547 high-risk population, the toxic dose proposed by our model and the therapeutic dose are  
548 identical.

549 For the healthy population, the time-frame for biomarker concentrations representing those of  
550 probable liver injury is around 2-18 hours. The dose threshold at which toxicity is predicted to  
551 be likely to occur is approximately 350 mg/kg. Note that this prediction is only slightly above  
552 the currently used toxic dose (300 mg/kg) [44]. The framework for a healthy population  
553 suggests that there is almost 100% chance of liver injury when the dose is only slightly higher  
554 than this amount.

555 For the fasted population, however, at the currently recommended therapeutic APAP dose (60  
 556 mg/kg), there is approximately 50% chance of liver injury progression. For any dose above  
 557 the known therapeutic threshold, liver injury progression is predicted to be highly probable  
 558 (above 60%); beyond approximately 2.5 h post-dose, HMGB1 concentrations remain  
 559 indicative of highly probable liver injury for the whole time-course. These results are  
 560 summarised in Table 4.

Population	Dose likely to induce liver injury	Time frame liver injury likely to occur
Healthy	>350 mg/kg	2-18 h
High-risk	>60 mg/kg	>2.5 h

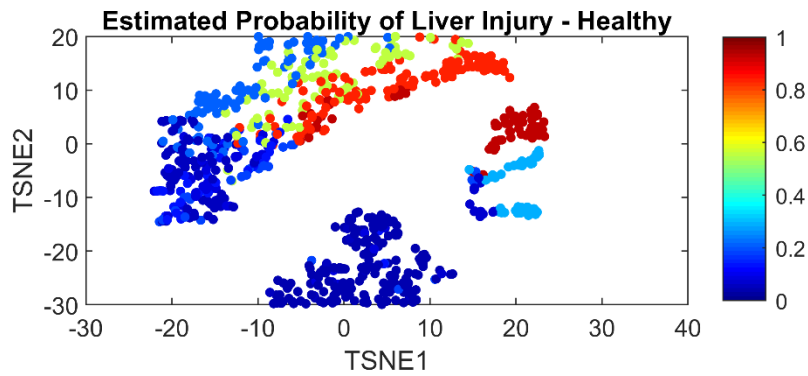
561 **Table 4: Critical dose and time ranges indicative of potential liver injury in both healthy**  
 562 **and high-risk populations.**

563 **3.6 Visualising the probability of liver injury following an APAP dose**

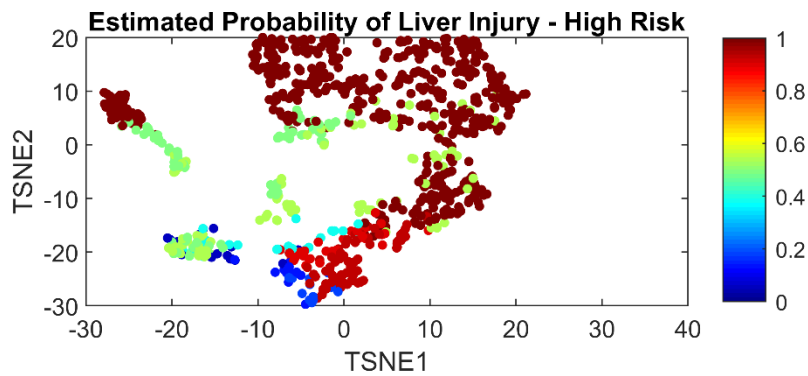
564 For each observation within the *in-silico* population, simulated HMGB1 concentrations, based  
 565 on our findings in [20], are used within the logistic regression model to determine the  
 566 corresponding probability of liver injury. Each *in-silico* observation is then visualised and  
 567 discriminated by their resultant liver injury probability using the TSNE method (Figure 9).  
 568 Relatively safe/unsafe observations are identifiable in both healthy and high-risk populations.  
 569 For the healthy population (Figure 9A) most of the observations have less than around 35%  
 570 chance of DILI progression. The small group of observations representing at-risk individuals  
 571 can be identified at the top, right-hand side of the image, portrayed by red markers. In stark  
 572 contrast, most of the *in-silico* observations have almost 100% chance of liver injury  
 573 progression in the high-risk population, as can be seen by the dominance of red markers in  
 574 Figure 9B. The observations corresponding to lower injury potential are indicated in the central  
 575 and the left-hand side of the plot.



A



B



576

577 **Figure 9: 2-dimensional TSNE visualisation of *in-silico* observations with respect to**  
578 **estimated probability of liver injury.** Separated liver injury probabilities are visualised for a  
579 range of *in-silico* generated inputs for both healthy (A) and high-risk (B) populations. DILI  
580 probabilities (0-1) for each individual observation are indicated by the colour bar.

581

#### 582 4 Discussion

583 The current clinical APAP framework is known to be inaccurate, mainly due to a dependency  
584 on known time of ingestion, but also due to inaccurate estimates of initial dose based on  
585 biomarkers currently criticised for being insensitive [25]. We previously reported a promising  
586 computational framework for predicting the probability of liver injury based on novel biomarker  
587 concentrations [20]. However, the identifiability analysis performed within this study highlights  
588 parameter unidentifiabilities within the model that required addressing prior to any further  
589 clinical application. Results of the analysis show where the original model required structural  
590 changes and also where more data was required in order to increase confidence in

591 predictions. Therefore, we have re-structured the original model where necessary and re-  
592 parameterised against additional data (from both fed and fasted mice). Identifiability analysis  
593 of the newly defined model structure found that only 7% of parameters are practically  
594 unidentifiable (compared with 24% previously).

595 As well as improving confidence in the mathematical representation of the system, we have  
596 here provided a model that is more representative of the clinical environment by including a  
597 wider range of APAP doses. Furthermore, the new treatment framework can now be adjusted  
598 for individuals considered to be at high risk of APAP-induced liver injury [3]. To account for the  
599 fact that many overdose patients have an increased underlying susceptibility to APAP-induced  
600 liver injury, we optimise our model against both fed and fasted mouse data. Factors that result  
601 in an APAP overdose patient being deemed “high-risk” such as alcohol consumption or  
602 malnutrition are known to deplete intracellular GSH stores, which subsequently increases their  
603 risk of potential toxicity [45]. Fasted animals have also been shown to have depleted GSH  
604 stores [46] and hence they are considered to be representative of high-risk individuals in our  
605 framework. When training the biomarker models against multiple datasets and comparing to  
606 the original model [20], which is calibrated against only 530 mg/kg APAP dose time-course  
607 data, there are noticeable adjustments in the parameter values, particularly for the GSH  
608 model. Many of these changes are intuitive and may be representative of changes in  
609 mechanisms due to fasting. Additionally, changes may be attributable to the fact that the model  
610 can now better account for a larger variety of dosing scenarios. The significant changes in  
611 parameter values highlight the importance of optimising against a range of both therapeutic  
612 and toxic datasets and both healthy and unhealthy populations.

613 The model we propose in this paper is more biologically relevant than our previous model [20].  
614 Originally, we assume that full and fragmented K18 have similar mechanisms and act as  
615 independent biomarkers. However, full and fragmented K18 are known to be effectively  
616 necrotic/apoptotic versions of the same biomarker [47]. Incorporating this into our model

617 framework we confirm that necrosis is the pre-dominant form of cell death in mice APAP  
618 overdose cases [48]. An identifiability analysis on the new model structure identifies an  
619 increased confidence in parameter estimates for the GSH, HMGB1, full and fragmented K18  
620 components of the model structure. There are, however, some unidentifiabilities remaining,  
621 particularly within the HMGB1 and fragmented K18 components of the model, indicating that  
622 additional data and model development is still required in order to have full confidence in the  
623 uniqueness of chosen mechanistic parameter values.

624 We provide a proof of-concept framework which can be used to make toxicity predictions for  
625 either a healthy, high-risk or mixed population. In practice, once a dose/time is determined  
626 using the multiple linear regression statistical model, probability of liver injury can be estimated  
627 based on the HMGB1-dependent logistic regression model, driven by PK simulations with the  
628 previously identified dose/time values. The range of doses for which the high-risk population  
629 is likely to suffer liver injury (based on predictions made within this *in-silico* framework) is  
630 extensive. Prediction accuracy of time since APAP administration and initial APAP dose is  
631 similar for the healthy and mixed populations, with a 3-4 h error for time since administration  
632 and a 65-75 mg/kg error for initial dose. The initial dose is much harder to predict in the high-  
633 risk population (184.1 mg/kg error). This result is unsurprising since a much larger range of  
634 doses will have a toxic effect if the liver is already impaired. Both Figure 8 and Table 4 show  
635 that in a high-risk population, any dose above approximately 60 mg/kg is highly likely to induce  
636 liver injury and therefore determining the exact dose in this scenario is difficult. Time since  
637 administration, however, could be predicted more accurately than in a healthy/mixed  
638 population (2.118 h error). It has already been found that amending treatment thresholds to  
639 account for high-risk individuals can better protect those with greater liver injury susceptibility  
640 [3]. Table 4 shows that liver injury in a healthy individual is predicted to occur within 2-18  
641 hours following an APAP dose. For a high-risk individual however, this likelihood is predicted  
642 to continue for the whole time-course investigated (24 hours). Results from this study further

643 endorse the idea that there are likely very different outcomes with respect to liver injury  
644 potential for healthy and high-risk individuals. More-informed decisions can therefore be made  
645 regarding optimal treatment if clinicians can identify those who are more susceptible to  
646 overdose. This would significantly improve patient outcomes while reducing the cost and  
647 burden of unnecessary antidote treatment.

648 The model was recalibrated to improve confidence in our approach and ensure that predictions  
649 are more clinically applicable prior to developing a clinical extension of the framework. The  
650 model has been calibrated against mouse data due to the quantity and quality of the toxicity  
651 data available, necessary for the rigorous development of a mathematical framework. Whilst  
652 metabolic similarities do exist between rodent and human (for example CYP2E1 expression),  
653 expression levels of enzymes such as CYP1A2 are known to vary across species [49],  
654 meaning direct translation of rodent-based models to the human case is rarely feasible and  
655 additional work may be required. Animal models account for the complex, physiological  
656 interplay within organisms, and therefore are often investigated during pharmacological  
657 research prior to application in humans. Although clinical APAP data is available, it is often  
658 sparse, erroneous and inherently highly variable. A Population-Pharmacokinetics (Pop-PK)  
659 approach could potentially provide insight into the stochasticity of the errors involved, but  
660 initially we have focused on the mouse framework as a convenient testing and development  
661 toolkit to determine efficacy in a well-controlled environment. The promising results of this  
662 study now provide confidence in the feasibility of translating our approaches to the human  
663 clinical case. Identifiability analysis has provided insight into areas within our model structure  
664 that remain uncertain, and therefore require additional consideration and improvement in order  
665 to enhance confidence in translated predictions. Regarding translation, all biomarkers used in  
666 the study can be measured in both animals and humans via the same methodologies.  
667 Inevitably, there are apparent differences between humans and mice such as the expression  
668 of metabolising enzymes, the mass APAP dose required to induce toxicity and the kinetics of

669 the biomarker profiles [44, 45]. However, the essential mechanistic processes of APAP  
670 metabolism, toxicity and action of the antidote are directly comparable. Our model accounts  
671 for a dose range 0-1000 mg/kg in mouse and this can be amended as a parameter within our  
672 model structure to be applicable to the clinical situation. In terms of the biomarkers used, they  
673 have now been measured in a time-dependent manner in multiple human studies [33, 41]. In  
674 its current form, our framework accounts for varied dose ranges and provides promise for  
675 clinical use in determining initial dose, time since ingestion and estimated probability of liver  
676 injury for both healthy and high-risk individuals. Further model development, integration of  
677 additional experimental data and translation to the clinical environment is now required to  
678 significantly advance this research to provide a state-of-the-art alternative to the methods  
679 currently used and considerably improve individualised treatment of APAP overdose.

#### 680 **Conflict of interest**

681 CLM is now an employee of AstraZeneca PLC.

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#### 689 **Author Contributions**

690 CLM contributed to the mathematical modelling and statistical analysis and wrote the  
691 manuscript; JL contributed to the mathematical modelling; ST and IJ contributed to the

692 statistical analysis; SDW contributed to the mathematical modelling and designed the  
693 research. All authors read and approved the final manuscript.

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