1 **A first-generation physiologically based pharmacokinetic (PBPK) model of alpha-**
2 **tocopherol in human influenza vaccine adjuvant**
3

4 Million A. Tegenge^a and Robert J. Mitkus^a
5

6 ^a Office of Biostatistics and Epidemiology, Center for Biologics Evaluation and Research, US
7 Food and Drug Administration, 10903 New Hampshire Ave, Silver Spring, MD 20993
8

9 **Running title:** PBPK model of alpha-tocopherol in human vaccines
10

11 **Corresponding author:** Million A. Tegenge, Office of Biostatistics and Epidemiology, Center
12 for Biologics Evaluation and Research, US Food and Drug Administration, 10903 New
13 Hampshire Ave, Silver Spring, MD 20993 e-mail: million.tegenge@fda.hhs.gov,
14 Phone: (01)-240-402-8802
15

16 **Key words:** alpha-tocopherol, emulsion, vaccines, adjuvants, AS03[®]
17
18
19

ABSTRACT

Alpha (α)-tocopherol is a component of a new generation of squalene-containing oil-in-water (SQ/W) emulsion adjuvants that have been licensed for use in certain influenza vaccines. Since regulatory pharmacokinetic studies are not routinely required for influenza vaccines, the *in vivo* fate of this vaccine constituent is largely unknown. In this study, we constructed a physiologically based pharmacokinetic (PBPK) model for emulsified α -tocopherol in human adults and infants. An independent sheep PBPK model was also developed to inform the local preferential lymphatic transfer and for the purpose of model evaluation. The PBPK model predicts that α -tocopherol will be removed from the injection site within 24 hours and rapidly transfer predominantly into draining lymph nodes. A much lower concentration of α -tocopherol was estimated to peak in plasma within 8 hours. Any systemically absorbed α -tocopherol was predicted to accumulate slowly in adipose tissue, but not in other tissues. Model evaluation and uncertainty analyses indicated acceptable fit, with the fraction of dose taken up into the lymphatics as most influential on plasma concentration. In summary, this study estimates the *in vivo* fate of α -tocopherol in adjuvanted influenza vaccine, may be relevant in explaining its immunodynamics in humans, and informs current regulatory risk-benefit analyses.

Introduction

Vaccination continues to play a highly important role in protecting and promoting public health. Numerous approaches have been exploited in the development of successful vaccines, and these involve the use of vaccine adjuvants. Adjuvants are components of certain vaccines that augment the magnitude and/or modulate the quality of the immune response (Koff et al. 2013; Levitz and Golenbock 2012; Pulendran and Ahmed 2011; Rappuoli 2011). Aluminum salts are the oldest and the most widely used vaccine adjuvants, but in recent years a new class of vaccine adjuvants has emerged: the squalene-containing oil-in-water (SQ/W) emulsion adjuvants. This class includes, among others, MF59[®] and AS03[®], which are components of certain influenza vaccines licensed by the European Medicines Authority (EMA) or the US Food and Drug Administration (USFDA) (Garcon et al. 2012; Gasparini et al. 2012; Levitz and Golenbock 2012; O'Hagan et al. 2011). Interest in the use of squalene-containing adjuvants has grown following the H1N1 pandemic as a means of sparing antigen while increasing immunogenicity.

AS03[®] is a new-generation squalene-containing emulsion adjuvant that contains α -tocopherol (11.86 mg) and squalene (10.69 mg) in the oil phase, emulsified by polysorbate 80 (4.86 mg), and a continuous aqueous phase of phosphate-buffered saline (Garcon et al. 2012). Alpha-tocopherol ($C_{29}H_{50}O_2$) is also the biologically active form of vitamin E, an essential, dietary lipid-soluble antioxidant responsible for protecting cell membranes against lipid peroxidation (Biesalski 2009). The precise mechanism by which SQ/W emulsions potentiate the immune response is not completely understood. However, experimental evidence with the adjuvant MF59[®] suggests enhanced immune cell recruitment, antigen uptake and upregulation of several cytokines and chemokines at the site of administration following intramuscular (IM) injection

(Calabro et al. 2011; Mosca et al. 2008; O'Hagan et al. 2012). Experimental studies in rodents and model-based pharmacokinetic studies in humans for SQ/W emulsions also suggest the absence of a local depot effect and rapid decay of the emulsion from the site of injection (Dupuis et al. 1999; Ott et al. 1995; Tegenge and Mitkus 2013). The addition of α -tocopherol into the oil phase of SQ/W emulsions has been shown to influence both the kinetics and the level of some cytokines at injection sites (Garcon et al. 2012; Morel et al. 2011).

The pharmacokinetics of α -tocopherol following oral administration has been extensively studied in humans (Blomstrand and Forsgren 1968; Ferslew et al. 1993; Jeanes et al. 2005; Kelleher and Losowsky 1968; Traber et al. 1994). Those studies have generally shown that α -tocopherol is mainly absorbed via the lacteals (lymphatic vessels in the small intestine), transported in plasma via lipoprotein binding, and cleared from plasma with a half-life of about 48 hr. However, the pharmacokinetic database for α -tocopherol solutions administered intramuscularly to humans appears to be limited to only two published studies. A study in premature neonates indicated that IM injection of α -tocopherol in a *non-emulsified* alcoholic solution peaked in plasma within four hours and was cleared slowly with a half-life of 44 hr (Colburn and Ehrenkranz 1983). The second study used α -tocopherol *acetate* in colloidal or olive oil solution for IM injection in premature neonates (Italian Collaborative Group on Preterm Delivery 1991). However, the emulsified alcohol, α -tocopherol, is the relevant form utilized in influenza vaccine adjuvants and its pharmacokinetics has not been studied experimentally. The former observation is crucial to adopting an appropriate modeling strategy, because the formulation in which α -tocopherol is delivered (e.g., oil-in-water vs. water-in-oil emulsion or alcoholic vs. oil solution), as well as the species of α -tocopherol (e.g., alcohol vs. ester) have been long known to significantly affect the

uptake of this compound into blood, as well as its tissue distribution (Bateman and Uccellini 1985; Njeru et al. 1994; Schmandke and Schmidt 1965).

Thus, we have carefully searched the literature database for the vaccine-relevant form of α -tocopherol (i.e. the alcohol form rather than ester) and formulation (i.e. O/W emulsion rather than free alcoholic or oil solution) and are aware of only two published pharmacokinetic studies, in any mammalian species, of α -tocopherol delivered in an O/W emulsion and administered IM, i.e., similar to the human vaccination scenario for influenza vaccines. Because the identified studies were conducted in sheep (Hidioglou and Karpinski 1991; Njeru et al. 1994), direct quantitative extrapolation of the results to the human situation would be highly uncertain. However, in such a scenario, physiologically-based pharmacokinetic (PBPK) modeling based on rational, well-informed assumptions can provide important predictions of the time course and tissue distribution in the relevant target species (humans), especially when experimental data in that species are lacking. In addition, since experimental pharmacokinetic studies in humans are not currently required for vaccine licensing across the world (Sun et al. 2012; WHO 2005; Wolf et al. 2010), PBPK models informed by relevant animal data serve as the basis for our predictions of the disposition of novel vaccine adjuvants (like MF59[®] and AS03[®]) in humans. Since they provide quantitative predictions of target concentration, they also in turn may inform the mechanism of action of these new products and contribute to a broader understanding of both benefit and risk in human subjects.

The purpose of this study was two-fold: 1) to develop a generic, flexible PBPK model for evaluation of the *in vivo* fate of novel vaccine adjuvants, and 2) to estimate the biodistribution of,

specifically, α -tocopherol, in humans following a single dose of squalene-containing adjuvant. We present the results of PBPK model predictions that describe the disposition of α -tocopherol in an SQ/W emulsion at the site of injection and draining lymph nodes in both human adults and infants. Furthermore, the PBPK model predicts the distal tissue distribution of α -tocopherol following injection of adjuvanted influenza vaccine. Finally, we compared vaccine-derived α -tocopherol concentration in human plasma and other tissues with that of background concentrations in plasma and selected tissue, as a useful first step in a quantitative risk analysis for this class of vaccine adjuvants.

Methods

PBPK model development and compound-specific assumptions

The generic PBPK model for vaccine adjuvant consisting of local and distal tissues is depicted in Figure 1A. Based on the adjuvant system AS03[®], the oil phase of the emulsion is composed of squalene and α -tocopherol. The oil phase is emulsified with the surfactant polysorbate 80 and has an average droplet size of about 180 nm (Garcon et al. 2012), comparable with the adjuvant MF59[®]. The PBPK model considered transport to both blood and draining lymph nodes from injection site muscle (Fig. 1B). However, because of the large size of the emulsion (Garcon et al. 2012), the type of local exposure (primarily interstitial during intramuscular injection), its highly lipophilic nature ($\log P=12$) ; Gershkovich and Hoffman 2005), and the known lymphatic absorption of oral α -tocopherol and lipophilic drugs as emulsions in association with chylomicrons (Blomstrand and Forsgren 1968), we assumed preferential transport into lymphatics, rather than into blood following local injection of emulsified α -tocopherol. The in vivo stability of O/W emulsions is largely unknown; however, limited cracking would be

expected following IM injection as a result of degradation or disassociation of the surfactant used to stabilize the emulsion (Bollinger 1970; Kalvodova 2010; van et al. 1999). Hence, we also incorporated an estimate of cracking of the emulsion (k_c) at the site of injection and within the draining lymph nodes similar to our recently published squalene model (Tegenge and Mitkus 2013).

The disposition of the emulsion in the injection site muscle and draining lymph nodes was described schematically (Fig. 1), as well as mathematically (Supplementary 1). Because of rapid esterase degradation of the polysorbate 80 in plasma (van Tellingén et al. 1999), we assumed that any emulsion that reached plasma would instantaneously crack, and the released α -tocopherol will then be transported primarily via binding to lipoproteins. The major lipoprotein that binds and transports α -tocopherol in plasma is low density lipoprotein (LDL) (Behrens et al. 1982; Biesalski 2009; Bjornson et al. 1976; Davies et al. 1969). Subsequent uptake of α -tocopherol into cells of target tissues is mainly via receptor-mediated endocytosis of LDL (Biesalski 2009; Thellman and Shireman 1985), while very limited diffusion of free α -tocopherol into or out of tissues can be expected based on its very high log P. Receptor-mediated uptake of α -tocopherol into selected tissues (liver, spleen, kidney and GIT) was modeled based on the uptake of radiolabeled LDL following binding to the LDL receptor in human tissues (Rudling et al. 1990). Distribution to the rest of the body was modeled for free α -tocopherol under flow-limited and well-stirred conditions. Each tissue compartment was described with a mass-balance differential equation that consisted of a series of tissue concentrations, flow rates and a partition coefficient. Detail of the model code and equations are displayed in Supplementary 1.

Physiological parameters

Based on the results of our previous model for emulsified squalene (Tegenge and Mitkus, 2013), lymphatic flow was modeled only for the injection site leading to the draining lymph nodes then to plasma. Lymphatic flow at the injection site was set at 0.2% of the rate of blood flow to injection site muscle and draining lymph nodes (Swartz 2001). Tissue volume for human adult (male mean body weight, BW= 73 kg) and 1 year old infant (mean BW=10 kg) were estimated based on ICRP89, Table 2.8 (ICRP 89 2002). We obtained the absolute value of cardiac output (CO) for both adult and infant (ICRP 89 2002). The % CO to each tissue in adult human (ICRP 89 2002) was used to estimate blood flow for both adult and infant and assumed a similar % CO for infant. Blood flow and tissue volume into deltoid muscle (adult model) and quadriceps muscle (infant model) were estimated based on published data (Evans et al. 1975; Potau et al. 2009; Radegran and Saltin 2000). Briefly, blood flows to deltoid and quadriceps muscle were estimated based on rates of 11.6 and 10.8 ml/g/min (Evans et al. 1975), respectively. Deltoid and quadriceps muscle volume were estimated based on 0.003 ml/g and 0.012 ml/g of body weight (Evans et al. 1975; Potau et al. 2009; Radegran and Saltin 2000), respectively. Physiological parameters for “total lymph nodes” were obtained from (ICRP 89 2002). Parameters for the draining lymph nodes were then predicted by assuming a proportional relationship between total lymph nodes in the adult human body ($n = \sim 650$) to that of upper armpit lymph nodes ($n = \sim 23$) (ICRP 89 2002; Tegenge and Mitkus 2013). Draining lymph node parameters (inguinal lymph) for the infant model were based on recent estimates from a computational lymphatic node model (Lee et al. 2013). Briefly, the inguinal lymph nodes volume for a 1-year-old infant were estimated by hybrid computational phantoms which use the number of lymphatic nodes recommendation from ICRP89 at different locations in human (Lee et al. 2013). For our

purpose, we directly used the reported inguinal lymph nodes (right or left) volume for a 1-year-old infant. The tissue:plasma partition coefficient (R_i) for α -tocopherol (log P=12) was estimated using the most recent algorithm method for highly lipophilic drugs (Poulin and Haddad 2012). Application of this algorithm to the estimation of R_i for squalene were recently reported (Tegenge and Mitkus 2013) and a similar method was employed for α -tocopherol. We used the same R_i value in both sheep and human models in the absence of species- and age-specific tissue neutral lipid equivalents, except for the R_i for adipose tissue (R_a) for sheep, which was optimized by the sheep PBPK model (discussed below). Physiological parameters for sheep (BW=39.5 kg) were taken from the standard literature (Craigmill 2003; Hay and Hobbs 1977; Potocnik and Wintour 1996; Talke et al. 2000; Upton 2008). Table 1 contains a summary of the physiological and physicochemical model inputs.

Biochemical parameters

The liver plays a major role in the metabolism of α -tocopherol. Previous studies have shown that CYP4F2 is the major CYP450 isoform involved in the metabolism of α -tocopherol (Parker et al. 2004; Sontag and Parker 2002; Traber 2010). In the absence of isoform-specific data in humans, *in vitro* Michaelis-Menten parameter values (k_m and V_{max}) obtained from rat microsomes were used to estimate CYP4F2-mediated metabolism of α -tocopherol in liver (Sontag and Parker 2002). Briefly, k_m was used as reported for rat; V_{max} was derived based on the only relevant *in vitro* studies in rat (Sontag and Parker 2002), adjusted for microsomal protein (45 mg per gram of liver) and liver weight (Lu et al. 2006), then allometrically scaled to humans and sheep ($BW^{0.75}$). Based on the expression of CYP4F2 in non-hepatic human tissues as compared to the liver (Nishimura et al. 2003), the level of metabolism by other tissues was considered negligible,

except for the kidney and GIT. For estimation of CYP4F2-mediated metabolism in the kidney and GIT, we used available human kidney and GIT CYP4F2 expression levels relative to the liver (Nishimura et al. 2003) and then normalized to weight of the tissue for estimation of V_{max} . LDL-receptor-mediated uptake of α -tocopherol into human tissue was estimated based on the observation that α -tocopherol is largely bound to lipoprotein in plasma and that LDL is the major binding plasma lipoprotein (Behrens et al. 1982; Bjornson et al. 1976; Davies et al. 1969). For this purpose k_{LDL} (LDL binding rate constant) was used as reported based on available human tissue uptake experiments using ^{125}I -labeled LDL (Rudling et al. 1990) and $B_{max\text{ LDL}}$ (maximum binding of ^{125}I -LDL) was normalized for tissue volume. The PBPK model included differential tissue uptake, based on studies that have shown a higher uptake of radiolabeled LDL in liver, spleen, kidney and GIT (Rudling et al. 1990). The (small) fraction of unbound α -tocopherol (f_u) was estimated from a previous study on the distribution of α -tocopherol in human plasma (Behrens et al. 1982). Emulsion cracking was mathematically described by first-order kinetics, and the cracking rate constant (k_c) was estimated following IM injection of an O/W emulsion in monkeys as previously described (Tegenge and Mitkus 2013). The summary of estimated biochemical parameters are displayed in Table 2.

Model parameter optimization and fitting

The serum concentration versus time profile of α -tocopherol in an O/W emulsion following IM injection was obtained from the only relevant study available in the literature (Njeru et al. 1994). Because that study was conducted in sheep (a species used historically for immunological research), it would not be appropriate to apply its plasma time course data directly to humans. Therefore, as an intermediate step, we constructed a PBPK model in sheep that was informed by

those published plasma data, in order to estimate the fractional lymphatic transfer of α -tocopherol (fl) following IM injection, rather than assuming 100%. Briefly, the serum (=plasma) concentration-time data points reported in Figure 4 of the sheep study (Njeru et al. 1994) were digitalized (using the open source software “Engauge Digitizer”) and fitted within the sheep PBPK model, while keeping all other parameters fixed. The initial value for fl was based on the assumption of preferential lymphatic transport of emulsified α -tocopherol, as described above and similar to our previous model for emulsified squalene (Tegenge and Mitkus 2013). Our preliminary optimization also required inclusion of adipose tissue-to-plasma partition (R_a) and inclusion of fraction unbound in plasma (fu). Model fitting was performed with the optimizer function in Vensim Professional® (Ventana Systems, Inc., Harvard, MA) by varying the initial value by a factor of up to 1000, depending on the parameter (Table 3). The optimum parameter value was identified based on the criteria of maximum payoff (defined as minimum error from experimental data). Additional literature data based on α -tocopherol acetate in an O/W emulsion were compiled for prediction of some target concentrations and evaluation of the sheep PBPK model (Hidioglou and Karpinski, 1991). In the absence of pharmacokinetic studies for α -tocopherol following IM injection of a SQ/W emulsion in humans or any known allometric relationship, the optimized fl value from the sheep PBPK model was used unchanged in the human PBPK model. Table 3 contains a summary of the optimized and final input parameters used in both the sheep and human PBPK models.

Simulation and uncertainty analysis

The sheep PBPK model was run using a single dose of 450 mg of α -tocopherol in O/W emulsion following IM injection into gluteal muscle (Njeru et al. 1994). The human PBPK model was

developed for 11.86 mg of α -tocopherol in a SQ/W emulsion following IM injection into deltoid muscle (adult) or quadriceps muscle (infant). This dose of α -tocopherol was comparable with the amount of α -tocopherol in the adjuvant system AS03[®] that is given as a single dose in a volume of 0.5 ml in influenza vaccine; although some clinical trials for children use a half-dose of α -tocopherol (Garcon et al. 2012). Thus, our PBPK model for infant was based on the maximum possible exposure following a single IM injection of vaccine.

An uncertainty analysis was performed to determine how variations in select model parameters (R_i , k_c , fl , k_p , fu , V_{max} , B_{max} , K_m) would affect the plasma concentration of α -tocopherol. For this purpose, a uniform distribution was assumed for all input parameters in the absence of known distributions for the individual parameters. The minimum and maximum values were set as $\pm 50\%$ of the value obtained from the literature. Monte Carlo simulations (1000) with Latin hypercube sampling, which allows for faster global sensitivity analysis of a large model, was performed using Vensim Professional[®]. The local sensitivity of the model output to change in input parameters was presented as a percentage change of the response variables (Shah and Betts 2012; Urva et al. 2010), as follows:

$$\%Change = \frac{100 \times (C_{\pm 50\%} - C_{sim})}{C_{sim}},$$

where C_{sim} refers to the simulated peak plasma concentration or area under plasma concentration (AUC) of α -tocopherol; $C_{\pm 50\%}$ is the peak concentration or AUC obtained following a 50% increase or decrease in the model parameter value.

Results

Sheep PBPK model predictions

Due to the scarcity of relevant experimental pharmacokinetic data for α -tocopherol administered IM in an O/W emulsion in any mammalian species, we initially developed a PBPK model of α -tocopherol in sheep (informed by limited published plasma time course data in that species), in order to estimate the important human model parameter, fl . The sheep PBPK model was constructed based on sheep-relevant physiological, anatomical and biochemical parameters (Tables 1-3). We first aimed at predicting the parameter, fl (fraction lymphatic), based on our earlier experience modeling a SQ/W emulsion. Previously we found that for a SQ/W emulsion a preferential lymphatic uptake of the emulsion droplet and free squalene with $fl=0.99$ seemed to reasonably predict the disposition time course of squalene from *mouse* quadriceps muscle (Tegenge and Mitkus 2013). In the absence of this parameter estimate for the adjuvant AS03[®], we used the initial estimate of $fl=0.99$ to predict the disposition of α -tocopherol in an O/W emulsion following gluteal muscle injection in *sheep*. Initially, we attempted to estimate the optimum value for fl while keeping all other parameters fixed; however, the model required inclusion of R_a (adipose:plasma partition coefficient) and fu (fraction unbound) to better fit the available plasma time course by visual inspection. Optimum parameters for fl , fu and R_a were, therefore, estimated by fitting the sheep PBPK model with the limited experimental pharmacokinetic data following IM injection of α -tocopherol in an O/W emulsion in sheep. Visual inspection of Figure 2A indicates that the optimized sheep PBPK model adequately predicted the experimental plasma-time profile of α -tocopherol from the literature (Njeru et al. 1994).

We further evaluated the sheep PBPK model by comparing its predictions with reported target concentrations following IM injection of the ester form (α -tocopherol *acetate*), in O/W in sheep

(Hidioglou and Karpinski, 1991). Firstly, we optimized the plasma-time concentration of α -tocopherol following IM injection of 1900 mg of α -tocopherol acetate (Supplementary 2). The plasma concentration-time profile from a second dataset of α -tocopherol acetate (2850 mg) was then compared with the PBPK model prediction (Hidioglou and Karpinski, 1991). As can be seen in Figure 2B, the concentration of α -tocopherol (from acetate form) peaked in plasma at 21 hr (Fig. 2 B) as compared to 9 hr for the alcohol form (Fig. 2A). Furthermore, the half-life of α -tocopherol in sheep plasma was 23 hr following IM injection of α -tocopherol vs. 82 hr following injection of α -tocopherol acetate. These differences could be in part due to limited esterase activity at the site of injection (muscle), which delayed the hydrolysis of the acetylated form (Hidioglou and Karpinski, 1991). Next, we used the PBPK model for predicting α -tocopherol concentration in distal sheep tissues (adipose, liver, kidney, and spleen) and compared with available literature data, again following α -tocopherol *acetate* injection (Hidioglou and Karpinski, 1991). For liver, spleen and kidney, 100% of the predicted concentrations were within 2 to 3-fold of the experimental results. Thus, given the variability in the experimental datasets, the PBPK model reasonably captured liver, kidney and spleen concentrations for the reported time points at each of the two doses (Fig. 2D-F). However, it was difficult to evaluate the predictive capacity of our PBPK model for adipose tissue since the experimental adipose tissue concentration reported by Hidioglou and Karpinski (1991) in sheep was only for omental fat, while our PBPK model (Fig. 2C) was based on the more comprehensive parameters of lumped adipose tissues (perirenal, epididymal and omental). Because of the significant difference in the pharmacokinetics of α -tocopherol following IM injection of the alcohol vs. ester form, we used the optimized *fl* value based on the alcohol form which is the most relevant for α -tocopherol in

human vaccines. The final input parameters used in sheep and human PBPK model are reported in Table 3.

Local disposition of α -tocopherol in humans

We used human-specific physiological and anatomical parameters in combination with the parameter estimate for fl from the optimized sheep PBPK model and the published human values for fu and R_a to construct a whole-body PBPK of α -tocopherol in humans following injection of the intact SQ/W emulsion. Hypothetically, following IM injection of vaccine containing the adjuvant AS03[®], there will be an intact O/W emulsion *in situ*, with the oil phase composed of α -tocopherol and squalene. Limited cracking, however, would be expected to result in some separation of the oil phase into free α -tocopherol and squalene, and this will affect the kinetics of the intact emulsion (Fig.1B).

A recent study based on the adjuvant MF59[®] supports the view that the intact SQ/W emulsion is responsible for triggering an immune response (Calabro et al. 2013), and this is likely to be the case for AS03[®] as well. Therefore, we considered it important to predict the disposition of the intact emulsion at the two potential sites of action in humans: muscle and draining lymph nodes. Here, the human PBPK model predicts the kinetics of both α -tocopherol in the intact emulsion and free α -tocopherol that was separated from the emulsion at the site of injection following cracking (Fig.3). Initially at time of injection, the predicted concentration of α -tocopherol in the intact SQ/W emulsion was two-fold higher in quadriceps (infant) than in deltoid (adult) muscle (Fig. 3A & B); this was driven by a smaller tissue volume in infants. However, the model also predicted a rapid exponential decay of intact SQ/W emulsion from both deltoid (adult) and

quadriceps (infant) muscles (Fig.3A & B). The removal of α -tocopherol in the SQ/W emulsion was described by a one-phase, exponential nonlinear curve, with a half-life of about 5 hr for both deltoid and quadriceps muscle. The predicted level of free α -tocopherol that leaked from the emulsion reached a peak at the site of injection at ~12 hr post dose and plateaued by 72 hr (Fig. 3C & D). Our model also predicted that α -tocopherol in a SQ/W emulsion will rapidly transfer to the draining lymph nodes (dLNs) where it will reach a maximum concentration within 24 hr post dose in both the adult and the infant models (Fig. 4A & B). The peak concentration of α -tocopherol in intact SQ/W emulsion in the dLNs was about three-fold higher for infant than adult model (Fig.4A & B), again due to smaller tissue volumes in infants. The concentration of free α -tocopherol in dLNs also peaked at ~12 hr as a result of cracking of the emulsion in draining lymph nodes and transfer of very low levels of free α -tocopherol from the injection site (Fig.4C & D).

Plasma pharmacokinetics and biodistribution of α -tocopherol in distal human tissues

The concentration of α -tocopherol in plasma was predicted to peak ~8 hr post-dose in both adult and infant PBPK models (Fig. 5A & B). At peak, plasma concentration of α -tocopherol was, 0.22 for adult and 1.24 ug/ml for infant (Fig. 5A & B, solid line). The area under plasma concentration curve (AUC) was 15.38 for adult and 96.98 ug x hr/ml for infant, which is equivalent to ~6-fold difference between infants and adults. The pharmacokinetics of α -tocopherol in human plasma was described by a mono-exponential decay in both age groups with a half-life of 19 hr for adults and 18.6 hr in infants. Because plasma concentration in infant appeared to trend slightly upward after 48 hr when unoptimized values of f_u and R_a were used, we asked whether this effect was model parameter dependent. Thus, the plasma concentration of

α -tocopherol was re-evaluated in both adult and infant using the sheep optimized fl , fu and R_a (Fig. 5A & B; broken lines). As can be seen, the plasma concentration of α -tocopherol appeared to decrease to almost negligible levels using the optimized sheep values, as a result of transfer and partition to adipose tissue (about 7 fold increased R_a). Furthermore, we asked whether the human PBPK model parameters could predict clearance of the background plasma concentration in humans within 24 hr consistent with the daily requirement of this essential dietary nutrient. Using human adult and infant model parameters we found that a peak plasma concentration of 11.3 ug/ml (adult) and 15 ug/ml (infant) α -tocopherol were essentially cleared within 24 hr (Supplementary 3A&B).

The predicted concentration of α -tocopherol peaked within 8 hr in liver, kidney, brain and spleen (Fig. 5C & D). However, the concentration of α -tocopherol in adipose tissue was predicted to reach a peak much later, at 48 hr (adult) and continue to slowly accumulate in infant (Fig. 5E & F). The transfer of α -tocopherol to the adipose tissue could be explained by its high lipophilicity and potential for binding to neutral lipids within adipocytes, similar to squalene (Tegenge and Mitkus 2013). Taken together, these results suggest that following IM injection of SQ/W emulsion in humans: 1) the highest concentrations of α -tocopherol will be in the draining lymph nodes, with much lower concentrations in plasma and distal tissues, and 2) adipose tissue will form a storage depot for exogenous α -tocopherol.

Preliminary risk analysis

As part of a preliminary risk analysis, we compared the injected dose and select predicted tissue concentrations of α -tocopherol following human vaccine adjuvant exposures in our model with

the recommended daily intake and background human tissue levels of α -tocopherol. For this purpose, we first compared the dose of α -tocopherol in an AS03-adjuvanted human vaccine (11.86 mg) with that of the recommended dietary allowances (RDA) developed by the Food and Nutrition Board (FNB) at The Institute of Medicine (IOM) of The National Academies (Institute of Medicine, 2000). The daily required levels of dietary α -tocopherol (RDA) for infants (7-12 months) and adults (>14 years) are 5 and 15 mg, respectively. The FNB has also promulgated tolerable upper intake levels (ULs) for children (1-3 years) and adult (>19 years) of 200 and 1000 mg, respectively. In the case of α -tocopherol, the ULs are maximum daily intake levels that are unlikely to cause adverse health effects from taking vitamin E supplements; there are no known adverse effects from α -tocopherol in food. Thus, a single nominal dose exposure from emulsified α -tocopherol in an adjuvanted vaccine for both infant and adult will be 17-84x lower than the nominal oral ULs for supplemental dietary α -tocopherol.

The FNB recommendations, however, are based on a different route of exposure (oral) with potentially variable absorption. Thus, we compared either National Academy of Sciences (NAS)-recommended or background plasma concentrations for this essential vitamin or the constitutive background tissue levels for α -tocopherol, with those of our PBPK model predictions. Specifically, we compared the peak concentrations of α -tocopherol following IM injection of a SQ/W emulsion in our model with the available human tissue background or plasma levels obtained from the literature (Dju et al. 1958). We found that the predicted percentage of vaccine-derived α -tocopherol in liver or adipose tissues is less than 7 or 25% of the respective background level of α -tocopherol reported for those tissues in infants and much lower in adults (Table 4). Parker (Parker 1988) has reported that the background adipose tissue levels of α -

tocopherol in adult human is in the range of 61-811 ug/g, which further confirms the minor contribution of vaccine-derived α -tocopherol to endogenous adipose tissue levels. Finally, the peak plasma concentration of vaccine-derived α -tocopherol was compared with the mean serum α -tocopherol background concentration for adults or infants. The ratio of peak vaccine-derived α -tocopherol to that of the background or recommended steady-state plasma concentrations in adults and infants was, again, very minute (<9%; Table 4). Furthermore, a bolus vaccine dose on top of background steady state plasma concentration did not alter the steady state behavior of α -tocopherol in human plasma (Supplementary 3C&D).

Uncertainty analyses

The uncertainty in the prediction of response variable, i.e., plasma concentration, a valid marker of systemic exposure, was explored graphically by varying selected model input parameters in the human adult model (Fig. 6 A&B). First, a global uncertainty analysis was performed without including fractional lymphatic transfer (fl), and the outer bounds of uncertainty (95% confidence bounds) show a maximum peak plasma concentration (C_{max}) of approximately 0.35 ug/ml at the end of the 1000 Monte Carlo simulations (Fig. 6A). We conducted an additional uncertainty analysis by running the model including fl among other parameters and set maximum value of fl to 100% (Fig. 6B). The C_{max} was approximately 0.8 ug/ml, which was about 3.6x higher than the 0.22 ug/ml model prediction and indicates a low likelihood that our model is under predicting peak plasma concentrations. This maximum theoretical upper bound value is still less than 1 ug/ml and a minute fraction of the recommended background human plasma concentration for adults (0.8 vs. 11.3 ug/ml). The uncertainty in the plasma concentration was highest at early time points and reached a minimum after ~120 hr post dose (Fig. 6A&B).

Next, we performed local sensitivity analyses in order to gain insight about which parameters are important for the uncertainty in the prediction of C_{max} and AUC. The greater the absolute value of the change, the more important the parameter became in affecting the response variable. Based on both C_{max} and AUC analyses, the most sensitive parameter was found to be the fraction unbound (f_u). For example, a 50% decrease in f_u yielded a 95% increase in C_{max} and 144% increase in plasma AUC (Fig. 6C). The adipose-to-plasma partition coefficient (R_a) was found to affect AUC but with less of an effect on C_{max} . A 50% decrease in R_a yielded about a 56% increase in AUC but only an ~20% decrease in C_{max} (Fig. 6C&D). A 100-fold reduction in R_a yielded only a 2-fold increase in C_{max} (from 0.22 to 0.5 ug/ml), again indicating the very limited sensitivity of plasma concentration estimates to R_a .

The biochemical parameters V_{max} , B_{max} , k_{mCYP} , and k_{LDL} had modest effects on AUC (Fig. 6C). However, C_{max} and AUC were essentially insensitive (<2 %) to changes in k_c , and changes in R_i values for, brain, liver, GIT, kidney or spleen (Fig. 6C &D). A local sensitivity analysis with 50% decrease of f_l resulted in more than a 115% increase in C_{max} but little effect on AUC. Increasing f_l to 100 % (maximum level) resulted in reduction of C_{max} by 85% and AUC by 70% (Fig. 6C&D). This result was not surprising given the optimized value for f_l used in our model (94%); since very little α -tocopherol is absorbed into plasma from muscle to begin within our model, small changes in f_l will lead to large changes in plasma concentration (on a percentage basis).

Discussion

Alpha-tocopherol (vitamin E) is an essential dietary nutrient, and several pharmacokinetic studies contribute to the general acceptance of the safety of dietary vitamin E even after high dose supplementation in adult humans (Biesalski 2009; Ferslew et al. 1993; Hall et al. 2005; Jeanes et al. 2005; Traber et al. 1994). However, the fate of α -tocopherol following IM injection of as a squalene-containing emulsion (AS03[®]) has not been studied in humans. In recent years, AS03[®] has gained more attention following its use in H1N1 pandemic influenza vaccines in certain countries and for its potential use in a pre-pandemic H5N1 influenza vaccine (Fox and Haensler 2013; Garcon et al. 2012). In this study, we developed the first PBPK model of α -tocopherol in a squalene-containing adjuvant in human influenza vaccines. Such a model may be useful to gain quantitative insight into the *in vivo* fate of the adjuvant in very young as well as adult humans, and the estimates provided by the model might then inform regulatory risk-benefit analyses for this new class of adjuvanted vaccines. Moreover, they might also help to better understand the mode-of-action of this new generation of vaccine adjuvants.

The current model is a multi-scale, physiologically-based mathematical model that takes into account several physicochemical and biochemical features of α -tocopherol, local preferential lymphatic uptake following injection, binding to plasma lipoproteins, CYP4F2-mediated metabolism and receptor-mediated endocytosis in tissues. The use of such multi-scale PBPK modeling is useful for predicting pharmacokinetic parameters and understanding pharmacodynamic properties of drugs (Eissing et al. 2011; Huang and Rowland 2012; Rowland et al. 2011). For the purpose of parameter estimation (*fl*) and partial model evaluation, we first

developed a PBPK model of α -tocopherol in an O/W emulsion following IM injection in sheep and optimized that model based on plasma-time course obtained from the only relevant mammalian study in the literature (Njeru et al. 1994; Hidioglou and Karpinski, 1991). The use of the sheep as a model species in pharmacokinetic studies of drugs is not common, but has been shown to be very relevant for the purpose of immunodynamic modeling especially with respect to lymphatic drainage of certain biologics like vaccine adjuvants (de Veer et al. 2012; deVeer et al. 2010). Based on the optimized sheep PBPK model, $f_l=0.94$ was used for the human PBPK model in the absence of any pharmacokinetic studies for α -tocopherol following IM injection of a SQ/W emulsion in humans or any known allometric relationship. By employing this optimized model parameter, our human PBPK model predicts that the concentration of α -tocopherol will peak in plasma within 8 hr following a single dose in both adult and infant (Fig. 5A&B). Also very low (<1.5 ppm), the concentration of α -tocopherol predicted in brain, liver, kidney, spleen or GIT in our study also peaked at 8 hr parallel to the plasma profile (Fig. 5C&D). The highest concentration of α -tocopherol was predicted to be in the draining lymph nodes (up to 300 ppm) following transport from the site of injection.

The PBPK model predicts that α -tocopherol will accumulate slowly in adipose tissue (up to 4 ppm by day 10, Fig. 5E&F), which appears to be reasonable for several reasons. Firstly, α -tocopherol is highly lipophilic (Abuasal et al. 2012; Gershkovich and Hoffman 2005); hence it has a very high adipose-to-plasma partition coefficient (Table 1). Secondly, previous studies from the oral route of exposure also showed preferential accumulation of α -tocopherol in adipose tissue (Novotny et al. 2012). Thirdly, in adipose tissue α -tocopherol was shown to be stored in deep adipocytes and specifically localized in the bulk lipid that forms lipid droplets (Traber and

Kayden 1987). Our PBPK model implicitly considers this distribution feature of α -tocopherol in adipose tissue since neutral lipid composition of adipose tissue was employed to predict the adipose-to-plasma partition coefficient used in the model (Poulin and Haddad 2012; Tegenge and Mitkus 2013). Overall the estimated high adipose-to-plasma partition coefficient and a corresponding higher adipose tissue concentration was consistent with the previously reported accumulation and slow turnover rate of α -tocopherol in human adipose tissue (Novotny et al. 2012; Traber, M.G. and Kayden 1987, Parker 1988). However, the percentage of vaccine-derived α -tocopherol relative to that of the background level in adipose tissue is still a minute fraction and not a concern (Table 4).

The disposition of the intact emulsion from the site of administration is relevant in potentially explaining the immunodynamics of commercial SQ/W emulsion adjuvants such as MF59[®] and AS03[®] since the administration site (muscle) and draining lymph nodes are major sites of action for this class of adjuvants (Garçon et al. 2012; O'Hagan et al. 2012). For MF59[®], studies have demonstrated that the whole emulsion but not the individual components (e.g., squalene alone) are responsible for the induction of an immune response, suggesting that *in vivo* delivery of a stable emulsion to the immune system is essential (Calabro et al. 2013; O'Hagan et al. 2012). In the case of AS03[®], characterization of the emulsion adjuvant in the presence or absence of α -tocopherol revealed significant differences in the kinetics and levels of certain cytokines and chemokines, suggesting that α -tocopherol itself has some immune modulating effect (Fox and Haensler 2013; Garçon et al. 2012; Morel et al. 2011). In our study, we predict the *in vivo* fate of α -tocopherol in the intact SQ/W emulsion as well as free α -tocopherol at the site of administration and in draining lymph nodes. Model simulation for deltoid and quadriceps

muscles and their draining lymph nodes showed that levels of α -tocopherol in the intact emulsion are up to 500-fold higher than free α -tocopherol, but that emulsified α -tocopherol will be essentially “cleared” from the site of injection within 24 hr. This 24-hour “window” is meaningful, since a recent experimental study with the adjuvant AS03[®] has indicated that antibody response to trivalent influenza split antigens was not changed when AS03[®] was injected in mice up to 24 hr prior to the antigen, but it decreased significantly after 48 hr (Morel et al. 2011). This observation, if true also for humans, is consistent with our PBPK model prediction of the disposition of the intact adjuvant at the site of injection.

The direct use of *fl* and certain other pharmacokinetic parameter estimates from animal studies within the human PBPK model and lack of experimental data to verify the human PBPK model are potential limitation of our model. Additionally, the infant model assumes similar tissue composition and blood flow as in human adult model. However, the use of nonclinical animal pharmacokinetic and mechanistic data to make predictions about the human situation is a mainstay of small-molecule and biologic drug development. In addition, we conducted several uncertainty analyses to identify the most influential model parameters and tested our model using the related acetate form of alpha-tocopherol, in order to better understand its limitations. The strength of our approach lies in its research into and innovative use of all available, relevant experimental, physiological, and physicochemical data in building the human PBPK model. In summary, the PBPK model constructed in this study provides quantitative insight into the *in vivo* fate and biodistribution of new-generation SQ/W emulsion vaccine adjuvants. As more experimental pharmacokinetic studies become available, a further refined model that also incorporates dose-response relationships for different age group could be developed in the future.

543

544 **Acknowledgment**

545 We thank Dr. Sorell Schwartz for helpful discussion throughout the development of the model
546 and Drs. Jason Aungst and Mark Levi for their review of our manuscript. This project was
547 supported in part by an appointment (M.A.T.) to the Research Participation Program at the
548 Center for Biologics Evaluation and Research administered by the Oak Ridge Institute for
549 Science and Education through an interagency agreement between the U.S. Department of
550 Energy and the U.S. Food and Drug Administration.

551

Figures

Figure 1. Schematic diagram of the whole body human PBPK model and hypothesized fate of emulsified α -tocopherol following IM injection in influenza vaccines. (a) Dashed line arrows indicate the direction of lymphatic uptake of α -tocopherol from site of administration to local draining lymph nodes then to plasma. The red lines denote LDL receptor-mediated uptake from plasma in selected tissues. (b) Hypothetically, following IM injection of SQ/W emulsion there will be intact emulsion with oil droplets comprised of squalene and α -tocopherol and limited cracking that results in some free squalene and α -tocopherol. Both the intact emulsion and free α -tocopherol (Free Toc) were assumed to preferentially transfer into draining lymph nodes vs. blood (see text). Moreover, binding of any free α -tocopherol to lipoproteins (LP) was assumed to occur instantaneously, while α -tocopherol from any intact SQ/W emulsion entering plasma will transfer rapidly into lipoproteins as a result of rapid cracking. Thick arrows indicate predominant reaction. The broken lines indicate rapid and instantaneous cracking of the emulsion in plasma. f_l (fraction of α -tocopherol that transfer into draining lymph nodes) and k_c (cracking rate constant for O/W emulsion).

Figure 2. Plasma concentration-time profile of α -tocopherol in sheep. (A) Concentration-time data from sheep (Njeru et al. 1994) were acquired and plotted together with the prediction from the sheep PBPK model (solid line) following IM injection of 450 mg of α -tocopherol in O/W emulsion. Triangles and squares represent experimental data extracted from the literature (Njeru et al. 1994). Note that the plasma concentration of α -tocopherol at $t=0$ was 0.7 ug/ml (background level) for both the experimental data and fitted curve. (B) Concentration-time data from sheep (Hidioglou and Karpinski, 1991) following IM injection of 1900 mg (rectangles) or 2850 mg (triangles) of α -tocopherol acetate were collected and plotted together with PBPK model predictions (solid, broken lines). (C-F) Geometric mean concentration of α -tocopherol in sheep adipose, liver, kidney and spleen following IM injection of 1900 mg (rectangles) or 2850 mg of α -tocopherol acetate (triangles) obtained from literature (Hidioglou and Karpinski, 1991) and PBPK model predictions (solid lines (1900 mg), broken lines(2850 mg)). Adipose tissue concentration (Hidioglou and Karpinski, 1991) was based on omental fat. Error bars represent coefficient variation (%) reported in (Hidioglou and Karpinski, 1991).

Figure 3. Human PBPK model prediction of removal of α -tocopherol from injection site following IM injection of SQ/W emulsion. (A-B) Injected α -tocopherol in the intact SQ/W emulsion rapidly removed from deltoid (adult) and quadriceps (infant) muscle within 24 hr. (C-D) Free α -tocopherol peaked in deltoid and quadriceps muscles within 24 hr.

Figure 4. Human PBPK model predicts rapid transfer of intact SQ/W emulsion into local dLNs. (A-B) α -tocopherol in the intact SQ/W emulsion rapidly transferred into local draining lymph nodes for both adult and infant models. (C-D) free α -tocopherol level gradually increases in dLNs as a result of emulsion cracking and transfer from the muscle compartment through lymphatic drainage.

Figure 5. Human PBPK model predictions of plasma and distal tissue biodistribution of α -tocopherol following IM injection of SQ/W emulsion. (A-B) Total plasma (free+bound) concentration-time profile of α -tocopherol in human adult and infant. Solid and broken curves are based on unoptimized and optimized parameters (f_l , f_u , and R_a), respectively. (C-D) α -tocopherol from injection of SQ/W emulsion peaked in liver, kidney, brain and spleen within 8 hr and did not accumulate in any of these tissues. (E-F) α -tocopherol slowly transfers into adipose tissue peaked within 48 hr (adult) and continue to slowly accumulate in infant.

Figure 6. Uncertainty in plasma concentration of α -tocopherol in the adult model. 50% and 95% confidence bounds for 1000 model simulations of 50% change in model parameters (A) without including fractional lymphatic transfer (f_l) or (B) including f_l and its maximum value was set to 100%. The blue curve was final PBPK model prediction of plasma concentration for human adult (see Fig. 5A). Literature derived or estimated parameters were changed individually by $\pm 50\%$ to assess their effect on (C) peak plasma concentration and (D) area under plasma concentration (AUC). R_i refer to tissue-to-plasma partition with subscript $i = a(\text{adipose}), b(\text{brain}), l(\text{liver}), g(\text{GIT}), s(\text{spleen}), k(\text{kidney})$; k_c (cracking rate constant for O/W emulsion); f_u (fraction unbound); $V_{maxCYP} = V_{max}$ value for CYP4F2 mediated metabolism of α -tocopherol; k_{LDL} = binding rate constant of ^{125}I into LDL-receptor; B_{maxLDL} = maximum binding of ^{125}I -LDL into its receptor; k_{mCYP} = apparent k_m for CYP4F2 mediated metabolism of α -tocopherol; V_{maxCYP}

= V_{max} value for CYP4F2 mediated metabolism of α -tocopherol. Since the maximum value of f_l and f_u are each 1, we cannot exceed that level during parameter optimization.

Reference List

- Abuasal, B.S., Qosa, H., Sylvester, P.W. and Kaddoumi, A. (2012) Comparison of the intestinal absorption and bioavailability of gamma-tocotrienol and alpha-tocopherol: in vitro, in situ and in vivo studies. *Biopharm. Drug Dispos.* 33, 246-256.
- Bateman, N.E. and Uccellini, D.A. (1985) Kinetics of D-alpha-tocopherol in a water soluble base in man. *J Pharm Pharmacol.* 37, 728-729.
- Behrens, W.A., Thompson, J.N. and Madere, R. (1982) Distribution of alpha-tocopherol in human plasma lipoproteins. *Am J Clin Nutr.* 35, 691-696.
- Biesalski, H.K. (2009) Vitamin E requirements in parenteral nutrition. *Gastroenterology* 137, S92-104.
- Bjornson, L.K., Kayden, H.J., Miller, E. and Moshell, A.N. (1976) The transport of alpha-tocopherol and beta-carotene in human blood. *J Lipid. Res* 17, 343-352.
- Blomstrand, R. and Forsgren, L. (1968) Labelled tocopherols in man. Intestinal absorption and thoracic-duct lymph transport of dl-alpha-tocopheryl-3,4- ^{14}C acetate dl-alpha-tocopheramine-3,4- ^{14}C dl-alpha-tocopherol-(5-methyl- 3H) and N-(methyl- 3H)-dl-gamma-tocopheramine. *Int. Z. Vitaminforsch.* 38, 328-344.
- Bollinger, J.N. (1970) Metabolic fate of mineral oil adjuvants using ^{14}C -labeled tracers. II. Mannide monooleate. *J Pharm Sci* 59, 1088-1092.
- Calabro, S., Tortoli, M., Baudner, B.C., Pacitto, A., Cortese, M., O'Hagan, D.T., De, G.E., Seubert, A. and Wack, A. (2011) Vaccine adjuvants alum and MF59 induce rapid recruitment of neutrophils and monocytes that participate in antigen transport to draining lymph nodes. *Vaccine.* 29, 1812-1823.
- Calabro, S., Tritto, E., Pezzotti, A., Taccone, M., Muzzi, A., Bertholet, S., De, G.E., O'Hagan, D.T., Baudner, B. and Seubert, A. (2013) The adjuvant effect of MF59 is due to the oil-in-water emulsion formulation, none of the individual components induce a comparable adjuvant effect. *Vaccine.* 31, 3363-3369.
- Colburn, W.A. and Ehrenkranz, R.A. (1983) Pharmacokinetics of a single intramuscular injection of vitamin E to premature neonates. *Pediatr Pharmacol. (New York)* 3, 7-14.
- Craigmill, A.L. (2003) A physiologically based pharmacokinetic model for oxytetracycline residues in sheep. *J Vet. Pharmacol. Ther* 26, 55-63.

646 Davies, T., Kelleher, J. and Losowsky, M.S. (1969) Interrelation of serum lipoprotein and
647 tocopherol levels. *Clin Chim. Acta* 24, 431-436.

648 de Veer, M., Kemp, J., Chatelier, J., Elhay, M.J. and Meeusen, E.N. (2012) Modulation of
649 soluble and particulate antigen transport in afferent lymph by monophosphoryl lipid A.
650 *Immunol. Cell Biol.* 90, 404-410.

651 deVeer, M., Kemp, J., Chatelier, J., Elhay, M.J. and Meeusen, E.N. (2010) The kinetics of
652 soluble and particulate antigen trafficking in the afferent lymph, and its modulation by
653 aluminum-based adjuvant. *Vaccine*. 28, 6597-6602.

654 Dju, M.Y., Mason, K.E. and Filer, L.J. (1958) Vitamin E (tocopherol) in human tissues from
655 birth to old age. *Am J Clin Nutr.* 6, 50-60.

656 Dupuis, M., McDonald, D.M. and Ott, G. (1999) Distribution of adjuvant MF59 and antigen gD2
657 after intramuscular injection in mice. *Vaccine*. 18, 434-439.

658 Eissing, T., Kuepfer, L., Becker, C., Block, M., Coboeken, K., Gaub, T., Goerlitz, L., Jaeger, J.,
659 Loosen, R., Ludewig, B., Meyer, M., Niederal, C., Sevestre, M., Siegmund, H.U.,
660 Solodenko, J., Thelen, K., Telle, U., Weiss, W., Wendl, T., Willmann, S. and Lippert, J.
661 (2011) A computational systems biology software platform for multiscale modeling and
662 simulation: integrating whole-body physiology, disease biology, and molecular reaction
663 networks. *Front. Physiol.* 2, 4.

664 Evans, E.F., Proctor, J.D., Fratkin, M.J., Velandia, J. and Wasserman, A.J. (1975) Blood flow in
665 muscle groups and drug absorption. *Clin Pharmacol. Ther* 17, 44-47.

666 Ferslew, K.E., Acuff, R.V., Daigneault, E.A., Woolley, T.W. and Stanton, P.E., Jr. (1993)
667 Pharmacokinetics and bioavailability of the RRR and all racemic stereoisomers of alpha-
668 tocopherol in humans after single oral administration. *J Clin Pharmacol.* 33, 84-88.

669 Fox, C.B. and Haensler, J. (2013) An update on safety and immunogenicity of vaccines
670 containing emulsion-based adjuvants. *Expert Rev Vaccines*. 12, 747-758.

671 Garcon, N., Vaughn, D.W. and Didierlaurent, A.M. (2012) Development and evaluation of
672 AS03, an Adjuvant System containing alpha-tocopherol and squalene in an oil-in-water
673 emulsion. *Expert Rev Vaccines*. 11, 349-366.

674 Gasparini, R., Amicizia, D., Lai, P.L. and Panatto, D. (2012) Aflunov((R)): a pre-pandemic
675 influenza vaccine. *Expert Rev Vaccines*. 11, 145-157.

676 Gershkovich, P. and Hoffman, A. (2005) Uptake of lipophilic drugs by plasma derived isolated
677 chylomicrons: linear correlation with intestinal lymphatic bioavailability. *Eur J Pharm*
678 *Sci* 26, 394-404.

679 Hall, W.L., Jeanes, Y.M. and Lodge, J.K. (2005) Hyperlipidemic subjects have reduced uptake
680 of newly absorbed vitamin E into their plasma lipoproteins, erythrocytes, platelets, and

681 lymphocytes, as studied by deuterium-labeled alpha-tocopherol biokinetics. *J Nutr.* 135,
682 58-63.

683 Hay, J.B. and Hobbs, B.B. (1977) The flow of blood to lymph nodes and its relation to
684 lymphocyte traffic and the immune response. *J Exp. Med* 145, 31-44.

685 Hidioglou, M. and Karpinski, K. (1991) Disposition kinetics and dosage regimen of vitamin E
686 administered intramuscularly to sheep. *Br. J Nutr.* 65, 465-473.

687 Huang, S.M. and Rowland, M. (2012) The role of physiologically based pharmacokinetic
688 modeling in regulatory review. *Clin Pharmacol. Ther* 91, 542-549.

689 ICRP 89. (2002) Basic anatomical and physiological data for use in radiological protection:
690 reference values. A report of age- and gender-related differences in the anatomical and
691 physiological characteristics of reference individuals. ICRP Publication 89. *Ann ICRP.*
692 32, 5-265.

693 Institute of Medicine, F.N.B. (2000) Dietary Reference Intakes: Vitamin C, Vitamin E,
694 Selenium, and Carotenoids. external link icon. In: Washington, DC: National Academy
695 Press.

696 Italian Collaborative Group on Preterm Delivery. (1991) Absorption of intramuscular vitamin E
697 in premature babies. *Dev. Pharmacol. Ther* 16, 13-21.

698 Jeanes, Y.M., Hall, W.L. and Lodge, J.K. (2005) Comparative (2)H-labelled alpha-tocopherol
699 biokinetics in plasma, lipoproteins, erythrocytes, platelets and lymphocytes in
700 normolipidaemic males. *Br. J Nutr.* 94, 92-99.

701 Kalvodova, L. (2010) Squalene-based oil-in-water emulsion adjuvants perturb metabolism of
702 neutral lipids and enhance lipid droplet formation. *Biochem. Biophys. Res Commun.* 393,
703 350-355.

704 Kelleher, J. and Losowsky, M.S. (1968) The absorption of vitamin E in man. *Biochem. J* 110,
705 20P-21P.

706 Koff, W.C., Burton, D.R., Johnson, P.R., Walker, B.D., King, C.R., Nabel, G.J., Ahmed, R.,
707 Bhan, M.K. and Plotkin, S.A. (2013) Accelerating next-generation vaccine development
708 for global disease prevention. *Science* 340, 1232-910.

709 Lee, C., Lamart, S. and Moroz, B.E. (2013) Computational lymphatic node models in pediatric
710 and adult hybrid phantoms for radiation dosimetry. *Phys. Med Biol.* 58, N59-N82.

711 Levitz, S.M. and Golenbock, D.T. (2012) Beyond empiricism: informing vaccine development
712 through innate immunity research. *Cell* 148, 1284-1292.

713 Lu, C., Li, P., Gallegos, R., Uttamsingh, V., Xia, C.Q., Miwa, G.T., Balani, S.K. and Gan, L.S.
714 (2006) Comparison of intrinsic clearance in liver microsomes and hepatocytes from rats

715 and humans: evaluation of free fraction and uptake in hepatocytes. *Drug Metab. Dispos.*
716 34, 1600-1605.

717 Morel, S., Didierlaurent, A., Bourguignon, P., Delhay, S., Baras, B., Jacob, V., Planty, C.,
718 Elouahabi, A., Harvengt, P., Carlsen, H., Kielland, A., Chomez, P., Garcon, N. and Van,
719 M.M. (2011) Adjuvant System AS03 containing alpha-tocopherol modulates innate
720 immune response and leads to improved adaptive immunity. *Vaccine*. 29, 2461-2473.

721 Mosca, F., Tritto, E., Muzzi, A., Monaci, E., Bagnoli, F., Iavarone, C., O'Hagan, D., Rappuoli,
722 R. and De, G.E. (2008) Molecular and cellular signatures of human vaccine adjuvants.
723 *Proc. Natl. Acad. Sci U S A*. 105, 10501-10506.

724 Nishimura, M., Yaguti, H., Yoshitsugu, H., Naito, S. and Satoh, T. (2003) Tissue distribution of
725 mRNA expression of human cytochrome P450 isoforms assessed by high-sensitivity real-
726 time reverse transcription PCR. *Yakugaku. Zasshi*. 123, 369-375.

727 Njeru, C.A., McDowell, L.R., Wilkinson, N.S., Linda, S.B., Rojas, L.X. and Williams, S.N.
728 (1994) Serum and tissue tocopherol in sheep after intramuscular injection and(or) dietary
729 vitamin E supplementation. *J Anim. Sci* 72, 739-745.

730 Novotny, J.A., Fadel, J.G., Holstege, D.M., Furr, H.C. and Clifford, A.J. (2012) This kinetic,
731 bioavailability, and metabolism study of RRR-alpha-tocopherol in healthy adults suggests
732 lower intake requirements than previous estimates. *J Nutr*. 142, 2105-2111.

733 O'Hagan, D.T., Ott, G.S., De, G.E. and Seubert, A. (2012) The mechanism of action of MF59 -
734 an innately attractive adjuvant formulation. *Vaccine*. 30, 4341-4348.

735 O'Hagan, D.T., Rappuoli, R., De, G.E., Tsai, T. and Del, G.G. (2011) MF59 adjuvant: the best
736 insurance against influenza strain diversity. *Expert Rev Vaccines*. 10, 447-462.

737 Ott, G., Barchfeld, G.L., Chernoff, D., Radhakrishnan, R., van, H.P. and Van, N.G. (1995)
738 MF59. Design and evaluation of a safe and potent adjuvant for human vaccines. *Pharm*
739 *Biotechnol*. 6, 277-296.

740 Parker, R.S. (1988) Carotenoid and tocopherol composition of human adipose tissue. *Am J Clin*
741 *Nutr*. 47, 33-36.

742 Parker, R.S., Sontag, T.J., Swanson, J.E. and McCormick, C.C. (2004) Discovery,
743 characterization, and significance of the cytochrome P450 omega-hydroxylase pathway
744 of vitamin E catabolism. *Ann N Y. Acad. Sci* 1031, 13-21.

745 Potau, J.M., Bardina, X., Ciurana, N., Camprubi, D., Pastor, J.F. and Barbosa, M. (2009)
746 Quantitative Analysis of the Deltoid and Rotator Cuff Muscles in Humans and Great
747 Apes. *Int. J. Primatol*. 30, 697-708.

748 Potocnik, S.J. and Wintour, E.M. (1996) Development of the spleen as a red blood cell reservoir
749 in lambs. *Reprod. Fertil. Dev*. 8, 311-315.

750 Poulin, P. and Haddad, S. (2012) Advancing prediction of tissue distribution and volume of
 751 distribution of highly lipophilic compounds from a simplified tissue-composition-based
 752 model as a mechanistic animal alternative method. *J Pharm Sci* 101, 2250-2261.

753 Pulendran, B. and Ahmed, R. (2011) Immunological mechanisms of vaccination. *Nat. Immunol.*
 754 12, 509-517.

755 Radegran, G. and Saltin, B. (2000) Human femoral artery diameter in relation to knee extensor
 756 muscle mass, peak blood flow, and oxygen uptake. *Am J Physiol. Heart. Circ. Physiol.*
 757 278, H162-H167.

758 Rappuoli, R. (2011) Twenty-first century vaccines. *Philos. Trans. R Soc. Lond. B Biol. Sci* 366,
 759 2756-2758.

760 Rowland, M., Peck, C. and Tucker, G. (2011) Physiologically-based pharmacokinetics in drug
 761 development and regulatory science. *Annu. Rev Pharmacol. Toxicol* 51, 45-73.

762 Rudling, M.J., Reihner, E., Einarsson, K., Ewerth, S. and Angelin, B. (1990) Low density
 763 lipoprotein receptor-binding activity in human tissues: quantitative importance of hepatic
 764 receptors and evidence for regulation of their expression in vivo. *Proc. Natl. Acad. Sci U*
 765 *S. A* 87, 3469-3473.

766 Schmandke, H. and Schmidt, G. (1965) [Studies on the resorption of alpha-tocopherol from oily
 767 and aqueous solutions]. *Int. Z. Vitaminforsch.* 35, 128-130.

768 Shah, D.K. and Betts, A.M. (2012) Towards a platform PBPK model to characterize the plasma
 769 and tissue disposition of monoclonal antibodies in preclinical species and human. *J*
 770 *Pharmacokinet. Pharmacodyn.* 39, 67-86.

771 Sontag, T.J. and Parker, R.S. (2002) Cytochrome P450 omega-hydroxylase pathway of
 772 tocopherol catabolism. Novel mechanism of regulation of vitamin E status. *J Biol. Chem*
 773 277, 25290-25296.

774 Sun, Y., Gruber, M. and Matsumoto, M. (2012) Overview of global regulatory toxicology
 775 requirements for vaccines and adjuvants. *J Pharmacol. Toxicol Methods* 65, 49-57.

776 Swartz, M.A. (2001) The physiology of the lymphatic system. *Adv. Drug Deliv. Rev* 50, 3-20.

777 Talke, P.O., Traber, D.L., Richardson, C.A., Harper, D.D. and Traber, L.D. (2000) The effect of
 778 alpha(2) agonist-induced sedation and its reversal with an alpha(2) antagonist on organ
 779 blood flow in sheep. *Anesth. Analg.* 90, 1060-1066.

780 Tegenge, M.A. and Mitkus, R.J. (2013) A physiologically-based pharmacokinetic (PBPK) model
 781 of squalene-containing adjuvant in human vaccines. *J Pharmacokinet. Pharmacodyn.* 40,
 782 545-556.

783 Thellman, C.A. and Shireman, R.B. (1985) In vitro uptake of [3H]alpha-tocopherol from low
 784 density lipoprotein by cultured human fibroblasts. *J Nutr.* 115, 1673-1679.

785 Traber, M.G. (2010) Regulation of xenobiotic metabolism, the only signaling function of alpha-
786 tocopherol? *Mol. Nutr. Food Res* 54, 661-668.

787 Traber, M.G. and Kayden, H.J. (1987) Tocopherol distribution and intracellular localization in
788 human adipose tissue. *Am J Clin Nutr.* 46, 488-495.

789 Traber, M.G., Ramakrishnan, R. and Kayden, H.J. (1994) Human plasma vitamin E kinetics
790 demonstrate rapid recycling of plasma RRR-alpha-tocopherol. *Proc. Natl. Acad. Sci U S.*
791 *A.* 91, 10005-10008.

792 Upton, R.N. (2008) Organ weights and blood flows of sheep and pig for physiological
793 pharmacokinetic modelling. *J Pharmacol Toxicol Methods* 58, 198-205.

794 Urva, S.R., Yang, V.C. and Balthasar, J.P. (2010) Physiologically based pharmacokinetic model
795 for T84.66: a monoclonal anti-CEA antibody. *J Pharm Sci* 99, 1582-1600.

796 van Tellingen O., Beijnen, J.H., Verweij, J., Scherrenburg, E.J., Nooijen, W.J. and Sparreboom,
797 A. (1999) Rapid esterase-sensitive breakdown of polysorbate 80 and its impact on the
798 plasma pharmacokinetics of docetaxel and metabolites in mice. *Clin Cancer Res* 5, 2918-
799 2924.

800 WHO. (2005) WHO guidelines on nonclinical evaluation of vaccines. WHO Technical Report
801 Series, 927.

802 Wolf, J.J., Kaplanski, C.V. and Lebron, J.A. (2010) Nonclinical safety assessment of vaccines
803 and adjuvants. *Methods Mol. Biol.* 626, 29-40.
804
805

806

807

Figure

(A)

(B)

Figure

Figure 3

Figure 4

Figure 5

Figure 6

Table 1. Physiological and physicochemical parameters used in the PBPK model.

<u>Tissue</u>	<u>Organ volume (L)</u>			<u>Blood flow (L/h)</u>			<u>Tissue-to-plasma partition</u>
	<u>Sheep</u>	<u>adult</u>	<u>Human infant</u>	<u>Sheep</u>	<u>adult</u>	<u>Human infant</u>	
Injection site ^a	0.500	0.219	0.120	2.726	1.524	0.835	3.3
Muscle ^b	10.942	28.781	1.780	60.779	11.959	11.462	3.3
Draining lymph nodes	0.001	0.003	0.001	0.033	0.121	0.022	3.2
Adipose	6.636	14.500	3.600	16.353	19.500	3.600	109.6 (759.2) ^c
Brain	0.079	1.450	0.950	3.434	46.800	8.640	9.3
GI tract	5.135	1.525	0.278	38.157	54.600	10.080	7.4
Liver	0.593	1.800	0.330	3.543	25.350	4.680	5.9
Kidney	0.119	0.310	0.070	17.443	74.100	13.680	4.3
Spleen	0.221	0.150	0.029	8.177	11.700	2.160	3.7
Plasma	0.978	3.000	0.400				
All other tissues	14.296	21.262	2.442	121.906	144.346	16.840	3.9

Physiological parameters were obtained from or estimated using standard literature (see Methods section) for sheep (39.5 kg), human adult (73 kg) and infant (10 kg). Tissue-to-plasma partition coefficients (R_i) were estimated according to the most recent algorithm for highly lipophilic molecules that is based on human neutral lipid composition [(Poulin and Haddad 2012).^aGluteal muscle (sheep), deltoid (adult human) and quadriceps (infant human); ^bOther skeletal muscle. ^chuman (optimized sheep) value.

Table 2. Estimated biochemical input parameters used in the PBPK model.

Parameter	Estimates	Species/Model System	Citation
k_c (h ⁻¹)	0.007	Monkey/ <i>in vivo</i>	(Tegenge and Mitkus, 2013)
$k_{m\ CYP}$ (uM)	42	Rat/ <i>in vitro</i>	(Sontag and Parker, 2002)
$V_{max\ CYP}$ (uM/h/Kg) ^a		Rat/ <i>in vitro</i>	(Sontag and Parker, 2002)
	Sheep 656.4		
	Human (adult model) 906.0		
	Human (infant model) 204.0		
k_{LDL} (ug/ml)	12	Human tissue/ <i>ex vivo</i>	(Rudling et al., 1990)
$B_{max\ LDL}$ ^b		Human tissue/ <i>ex vivo</i>	(Rudling et al., 1990)
	Kidney 1.1		
	Spleen 1.1		
	Liver 0.8		
	GI tract 0.5		

^aestimates are for liver and the value for kidney (25%) and GIT(6%) relative to liver based on mRNA expression level of CYP4F2 (Nishimura et al. 2003). ^breceptor-mediated uptake was estimated for tissue with B_{maxLDL} greater than 0.1(Rudling et al., 1990) and normalized for tissue volume. k_c =cracking rate constant for O/W emulsion; k_{LDL} = binding rate constant of ¹²⁵I to LDL-receptor; B_{maxLDL} =maximum binding of ¹²⁵I-LDL to its receptor; k_{mCYP} =apparent k_m for CYP4F2-mediated metabolism of α -tocopherol; V_{maxCYP} = V_{max} value for CYP4F2 mediated metabolism of α -tocopherol.

Table 3. Input parameters estimated from the PBPK model.

<u>Parameter</u>	<u>Initial value</u>	<u>Optimization range</u>	<u>Model estimates (optimized)</u>		
			<i>Sheep</i>	<i>Human(adult)</i>	<i>Human (infant)</i>
<i>fl</i> ^a	0.99	0.099 -1.0	0.94 ^b	0.94	0.94
<i>fu</i>	0.12 ^c	0.0012-1.0	0.08	0.12 ^c	0.12 ^c
<i>R_a</i>	109.6 ^d	10.96-1096	759.2	109.6	109.6

^ainitial value based on the assumption of preferential lymphatic uptake similar to squalene model estimated for the adjuvant MF59 (Tegenge and Mitkus, 2013). ^boptimized value informed by published plasma time course data in sheep(Njeru et al. 1994). ^cestimated from the binding of α -tocopherol to human plasma lipoprotein (Behrens et al. 1982). Since the maximum value of *fl* (fraction of α -tocopherol that transfers into lymphatics) and *fu* (fractional unbound in plasma) are each 1, neither value can exceed that level during parameter optimization. ^d*R_a*: adipose tissue-to-plasma partition coefficient for humans.

Table 4. Comparison of α -tocopherol peak concentration from single IM injection of SQ/W emulsion with that of endogenous concentration in human tissues.

	Vaccine (ug/g) ^a		Human tissues (ug/g) ^b		% Vaccine/endogenous	
	Adult	Infant	Adult	Infant	Adult	Infant
Liver	0.16	0.88	25.00 ^b	9.00 ^b	0.64	6.70
Adipose	0.65	4.10	245.00 ^b	17.00 ^b	0.27	24.11
Plasma	0.22	1.24	11.30 ^c	15.00 ^d	1.95	8.27

^aPeak tissue concentration predicted from human PBPK model following IM injection of SQ/W emulsion that contain 11.86mg of α -tocopherol. ^bLiterature data for nine infants (ages 1 to 8 months) and two adult (ages 23 to 31 years) were used to calculate average human liver and adipose tissue background concentration of α -tocopherol (Dju et al., 1958). ^cMean serum vitamin E concentration for men (31-50 years) obtained from NAS (Institute of Medicine 2000). ^dMean serum level recommended for human infant (Biesalski, 2009).