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Gudmand-Høyer, Johanne; Ottesen, Johnny T.

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# Analysis and validation of a new extended method for estimating plasma free cortisol including neutrophil elastase and competition from other steroids

Johanne Gudmand-Hoeyer[a] and Johnny T. Ottesen[a,*]

[a] *Department of Science and Environment, Roskilde University,*
[*] Corresponding author. *E-mail address:* johnny@ruc.dk, *Postal address:* Department of Science and Environment, Roskilde University, Building 27.1, 4000 Roskilde, Denmark

**Abstract**

A non-linear mechanistic model for the distribution of cortisol in plasma on free and bound forms is proposed. The influence of progesterone, testosterone and neutrophil elastase on the cortisol distribution in the blood is investigated.

The activity of neutrophil elastase is directly included in the model with the concentration of elastase and the kinetic constants describing the activity of elastase collected in one single input variable. The model is very sensitive towards this input variable and fits data excellently, when it is allowed to be subject specific.

The analysis shows that steroids such as testosterone with low affinity for corticosteroid-binding globulin (CBG) do not significantly influence the concentration of free cortisol. Progesterone has a high affinity for CBG, but low plasma concentrations compared to cortisol. Contrary to expectations, progesterone is shown to impact the distribution of cortisol in plasma both under circumstances with high levels as seen in pregnancy and during the normal menstrual cycle of women.

Comparing the predictions of our model with predictions made with the equilibrium models by Coolens et al. [1], Dorin et al. [2] and Nguyen et al. [3] shows that the models differ considerably not only in their predictions for free cortisol, but also for cortisol on bound forms; i.e. bound to albumin, intact CBG and elastase-cleaved CBG.

Disregarding some of the smallest terms of the model equations a reduced version of the model in form of a fourth order polynomial equation is

obtained. The reduced version of the model performs almost identically to the full version and serves as a new formula for calculating the plasma free cortisol concentration.

*Keywords:*
Cortisol, Corticosteroid-Binding Globulin, Mechanism based model, Progesterone, Testosterone, Neutrophil elastase

## 1. Introduction

The steroid hormone cortisol is found in blood bound to the transport proteins corticosteroid-binding globulin (CBG), albumin (Alb), and in the free form with a distribution of 70%, 20% and 10%, respectively [4]. Although free and bound cortisol contribute to the total concentration of cortisol in blood, only free cortisol is considered bioactive [5, 4]. By equilibrium dialysis, gel filtration or ultrafiltration direct measurement of free cortisol in human plasma can be done. However, the methods are both time-consuming and labour-intensive [6]. Thus, in most clinical procedures for measuring cortisol levels only the total cortisol concentration is measured [4]. Afterwards conservation and equilibrium assumptions are used to calculate concentrations of free and bound cortisol [3, 2].

The most often used formula for calculating free cortisol is the Coolens formula, which includes solving a second order polynomial [1, 2]. Coolens et al. [1] considered cortisol, CBG and albumin only and assumed the relevant reactions to be in equilibrium as well as conservation of the corresponding substances. Furthermore, the ratio of total albumin to its affinity for cortisol was assumed constant. Later Dorin et al. [2] developed an improved, cubic model with total albumin and its affinity for cortisol included as input variable and parameter, respectively, and by this excellent work demonstrated the importance of albumin concentration in cases with combined albumin and CBG deficiencies [2]. Nguyen et al. [3] extended the model further by considering two states of CBG: high-affinity, native CBG and low-affinity, elastase-cleaved CBG ($CBG^*$), assuming equilibrium of the relevant reactions and conservation of the total amounts of the corresponding substances. This was elegantly generalized to a fourth order formula [3]. In their formula Nguyen et al. [3] ignored the actual enzymatic reaction cleaving CBG into $CBG^*$ and instead took the total amounts of each of CBG and $CBG^*$ to be conserved.

Despite a high affinity for progesterone and a relatively high affinity for testosterone [7], the binding of progesterone and testosterone to CBG is often disregarded [1, 2, 3]. However, the concentrations of these two hormones varies considerably under both normal physiological and patophysiological circumstances. There are concentration differences between women and men [8, 9] and for women during the menstrual cycles [10, 11] and pregnancy [12, 13]. For many women with polycystic ovaries or hirsutism increased levels of testosterone are observed [14].

In this paper we expand on the equilibrium considerations of cortisol's distribution in the blood by including testosterone and progesterone competing with cortisol in binding with CBG and albumin. In contrast to earlier work [1, 2, 3], we include the enzymatic elastase reaction transforming native CBG into CBG$^*$. The resulting equilibrium model can with small reductions be stated as a fourth order polynomial, which may serve as a new formula for calculating free cortisol.

The goal of this paper is to 1) make an improved formula for calculating free cortisol, 2) quantify the amount of cortisol binding to proteins in the bloodstream competing with other steroid hormones, 3) investigate the influence of neutrophil elastase, 4) compare the predictions made by the proposed models to prior models by Coolens et al. [1], Dorin et al. [2], Nguyen et al. [3] under separate physiologically relevant circumstances, and 5) investigate and discuss the impact of variation in parameter and input variable values resulting from an intensive literature study.

## 2. Methods

### *2.1. Model development*

#### *2.1.1. The bio-chemistry of plasma cortisol concentration*

In this section we sketch the reactions of the bio-chemical system controlling cortisol's dynamic in the blood and name the variables included in the mathematical description in the following section 2.1.2. The chemical reactions are written schematically in box 2.1.1.

Let $X_F$ denote the concentration of free cortisol (Cort) in blood, $X_C$ that of free native CBG, $X_{FC}$ that of cortisol bound to native CBG (Cort:CBG), $X_A$ the free albumin concentration in blood, and $X_{FA}$ that of cortisol bound to albumin (Cort:Alb).

During inflammation neutrophils may raise the level of neutrophil elastase (NE) in the blood. Elastase acts as an enzyme in transforming high-affinity,

native CBG into a state CBG$^*$ with lower affinity [15, 16, 17, 18]. Elastase causes an irreversible change in the reactive centre loop of CBG changing the conformational state of the protein from a S-state (S for stressed) into a R-state (R for relaxed), thus lowering the affinity by approximately a factor 10 [19, 20, 21]. By $X_{C^*}$ we denote the concentration of free CBG$^*$, $X_{FC^*}$ that of cortisol bound to CBG$^*$ (Cort:CBG$^*$), $X_E$ that of free elastase, and $X_{CE}$ that of elastase bound to CBG (CBG:NE).

The enzymatic conversion of CBG into CBG$^*$ is assumed to follow Michaelis-Menten kinetics with $k_{CE}^+$ and $k_{CE}^-$ being the association and dissociation rates, respectively, of the reversible CBG:NE complex-binding and $k_{C^*}^+$ the catalytic rate describing the irreversible conversion of CBG:NE into CBG$^*$.

By $X_T$ and $X_P$ we denote the concentration of testosterone (Tes) and progesterone (Prog), respectively, $X_{TC}$ and $X_{PC}$ those of testosterone and progesterone bound to CBG (Tes:CBG and Prog:CBG), respectively, ignoring potential binding to CBG$^*$. Similar, $X_{TA}$ and $X_{PA}$ denote concentrations of testosterone and progesterone bound to albumin (Tes:Alb and Prog:Alb), respectively.

By $k_{Fs}$ we denote the secretion rate of cortisol into the bloodstream by the adrenal glands. The clearing rate of cortisol will be denoted $k_{Fe}$. Secretion and elimination of CBG will be denoted $k_{Cs}$ and $k_{Ce}$, respectively, whereas $k_{C^*e}$ denote the clearing rate of CBG$^*$. The fast production rate, $k_{Es}$, for elastase and elimination rate, $k_{Ee}$, are also considered. Clearing rates for the other substances will be ignored since they are supposed to be much smaller [22].

The association and dissociation rate constants of steroids and their transport proteins are denoted with a plus and a minus, respectively, e.g. the association and dissociation rate constants of cortisol and CBG are denoted $k_{FC}^+$ and $k_{FC}^-$, respectively. We use the symbol $\emptyset$ for the pool of substances, commonly known as sinks or sources, assuming that the concentrations of substances in this pool do not affect the system.

- Cort+CBG $\underset{k_{FC}^{-}}{\overset{k_{FC}^{+}}{\rightleftharpoons}}$ Cort:CBG
- Cort+CBG$^*$ $\underset{k_{FC^*}^{-}}{\overset{k_{FC^*}^{+}}{\rightleftharpoons}}$ Cort:CBG$^*$
- Cort+Alb $\underset{k_{FA}^{-}}{\overset{k_{FA}^{+}}{\rightleftharpoons}}$ Cort:Alb
- $\emptyset \xrightarrow{k_{Fs}}$ Cort $\xrightarrow{k_{Fe}} \emptyset$
- $\emptyset \xrightarrow{k_{Cs}}$ CBG $\xrightarrow{k_{Ce}} \emptyset$
- Tes+CBG $\underset{k_{TC}^{-}}{\overset{k_{TC}^{+}}{\rightleftharpoons}}$ Tes:CBG
- Tes+Alb $\underset{k_{TA}^{-}}{\overset{k_{TA}^{+}}{\rightleftharpoons}}$ Tes:Alb
- Prog+CBG $\underset{k_{PC}^{-}}{\overset{k_{PC}^{+}}{\rightleftharpoons}}$ Prog:CBG
- Prog+Alb $\underset{k_{PA}^{-}}{\overset{k_{PA}^{+}}{\rightleftharpoons}}$ Prog:Alb
- CBG + NE $\underset{k_{CE}^{-}}{\overset{k_{CE}^{+}}{\rightleftharpoons}}$ CBG:NE $\xrightarrow{k_{C^*}^{+}}$ CBG$^*$ + NE
- CBG$^*$ $\xrightarrow{k_{C^*e}} \emptyset$
- $\emptyset \xrightarrow{k_{Es}}$ NE $\xrightarrow{k_{Ee}} \emptyset$

Box 2.1.1 The reactions of the bio-chemical system controlling cortisols dynamic in the blood including the dynamics of cortisol (Cort), Corticosteroid-binding globulin on uncleaved and cleaved form (CBG and CBG$^*$, respectively), albumin (Alb), progesterone (Prog), testosterone (Tes), and neutrophil elastase (NE). The small $k_s$ denote the rate constants of the reactions. A common symbol $\emptyset$ denotes the sources of CBG, Cort, and NE and elimination products of CBG, CBG$^*$, Cort, and NE, since the concentrations of substances in this pool are assumed not to affect the system. The potential binding of Prog and Tes to CBG$^*$ is ignored as well as the synthesis and clearing of Alb, Prog and Tes.

*2.1.2. A static model by equilibrium considerations*

Assuming law of mass action and an equilibrium between the elimination and enzymatic synthesis of CBG$^*$ gives,

$$k_{C^*e} \cdot X_{C^*} = k^+_{C^*} \cdot X_{CE} \tag{1}$$

Making a standard quasi steady state approximation, where the concentration of CBG:NE is assumed constant compared to the changes in the concentrations of other species, gives,

$$X_{CE} = \frac{k^+_{CE}}{k^-_{CE}+k^+_{C^*}} \cdot X_E \cdot X_C \tag{2}$$

Combining equation 1 and 2 results in,

$$X_{C^*} = \frac{k^+_{C^*}}{k_{C^*e}} K^{-1}_{M_{CE}} \cdot X_E \cdot X_C \tag{3}$$

where $K_{M_{CE}}$ is the Michaelis-Menten constant $K_{M_{CE}} = \frac{k^-_{CE}+k^+_{C^*}}{k^+_{CE}}$.

From equation 3 it follows that the fraction $X_{C^*}/X_C$ becomes proportional to $X_E$,

$$\frac{X_{C^*}}{X_C} = \frac{X_E}{K_{CE}} \tag{4}$$

with an approximated 'equilibrium dissociation constant' $K_{CE} = \frac{k_{C^*e}}{k^+_{C^*}} K_{M_{CE}}$.

Using the law of mass action and assuming equilibrium we obtain ordinary equilibrium relations from the rest of the chemical reactions,

$$X_F \cdot X_C = K_{FC} \cdot X_{FC} \tag{5a}$$
$$X_F \cdot X_{C^*} = K_{FC^*} \cdot X_{FC^*} \tag{5b}$$
$$X_F \cdot X_A = K_{FA} \cdot X_{FA} \tag{5c}$$
$$X_T \cdot X_C = K_{TC} \cdot X_{TC} \tag{5d}$$
$$X_T \cdot X_A = K_{TA} \cdot X_{TA} \tag{5e}$$
$$X_P \cdot X_C = K_{PC} \cdot X_{PC} \tag{5f}$$
$$X_P \cdot X_A = K_{PA} \cdot X_{PA} \tag{5g}$$

with $K_{FC} = \frac{k^-_{FC}}{k^+_{FC}}$, $K_{FC^*} = \frac{k^-_{FC^*}}{k^+_{FC^*}}$, $K_{FA} = \frac{k^-_{FA}}{k^+_{FA}}$, $K_{TC} = \frac{k^-_{TC}}{k^+_{TC}}$, $K_{TA} = \frac{k^-_{TA}}{k^+_{TA}}$, $K_{PC} = \frac{k^-_{PC}}{k^+_{PC}}$, $K_{PA} = \frac{k^-_{PA}}{k^+_{PA}}$ being the equilibrium dissociation constants of the respective reactions.

In addition to equations (4)-(5g), we have three source-sink balances,

$$k_{Fs} = k_{Fe} \cdot X_F \tag{6a}$$
$$k_{Cs} = k_{Ce} \cdot X_C \tag{6b}$$
$$k_{Es} = k_{Ee} \cdot X_E \tag{6c}$$

which are not used for finding the concentrations, but are needed in order to estimate the fractions between source and elimination constants at steady state.

Assuming conservation of total concentrations of the involved substances in addition to the above equilibrium assumptions, we obtain five coupled second order algebraic equations in five variables,

$$X_{C0} = X_C + X_{C^*} + X_{FC} + X_{FC^*} + X_{TC} + X_{PC} + X_{CE} \tag{7a}$$
$$= X_C \left(1 + \frac{X_E}{K_{CE}} + \frac{X_E}{K_{M_{CE}}} + \frac{X_F}{K_{FC}}(1 + \frac{X_E K_{FC}}{K_{CE} K_{FC^*}}) + \frac{X_T}{K_{TC}} + \frac{X_P}{K_{PC}}\right) \tag{7b}$$
$$X_{F0} = X_F + X_{FC} + X_{FC^*} + X_{FA}$$
$$= X_F \left(1 + \frac{X_C}{K_{FC}}(1 + \frac{X_E K_{FC}}{K_{CE} K_{FC^*}}) + \frac{X_A}{K_{FA}}\right) \tag{7c}$$
$$X_{T0} = X_T + X_{TC} + X_{TA}$$
$$= X_T \left(1 + \frac{X_C}{K_{TC}} + \frac{X_A}{K_{TA}}\right) \tag{7d}$$
$$X_{A0} = X_A + X_{FA} + X_{TA} + X_{PA}$$
$$= X_A \left(1 + \frac{X_F}{K_{FA}} + \frac{X_T}{K_{TA}} + \frac{X_P}{K_{PA}}\right) \tag{7e}$$
$$X_{P0} = X_P + X_{PC} + X_{PA}$$
$$= X_P \left(1 + \frac{X_C}{K_{PC}} + \frac{X_A}{K_{PA}}\right) \tag{7f}$$

with $X_{C0}$ being the total amount of CBG and CBG$^*$, $X_{F0}$ of cortisol, $X_{T0}$ of testosterone, $X_{A0}$ of albumin, and $X_{P0}$ of progesterone in all forms.

By using relative variables $x_C = X_C/X_{C0}$, $x_F = X_F/X_{F0}$, $x_T = X_T/X_{T0}$, $x_A = X_A/X_{A0}$ and $x_P = X_P/X_{P0}$ and grouping the parameters, the following

dimensionless form in five variables and with 13 parameters is obtained,

$$p_0 x_C + p_{CF} x_C x_F + p_{CT} x_C x_T + p_{CP} x_C x_P = 1 \tag{8a}$$
$$x_F + p_{FC} x_F x_C + p_{FA} x_F x_A = 1 \tag{8b}$$
$$x_T + p_{TC} x_T x_C + p_{TA} x_T x_A = 1 \tag{8c}$$
$$x_A + p_{AF} x_A x_F + p_{AT} x_A x_T + p_{AP} x_A x_P = 1 \tag{8d}$$
$$x_P + p_{PC} x_P x_C + p_{PA} x_P x_A = 1 \tag{8e}$$

where $p_0 = 1 + \frac{X_E}{K_{CE}} + \frac{X_E}{K_{M_{CE}}} = 1 + \frac{X_E}{K_{CE}}(1 + \frac{k_{C^*e}}{k^+_{C^*}}) \approx 1 + \frac{X_E}{K_{CE}} \approx 2.0$, $p_{FC} = \frac{X_{C0}}{K} \approx 19.9$, $p_{FA} = \frac{X_{A0}}{K_{FA}} \approx 2.0$, $p_{TC} = \frac{X_{C0}}{K_{TC}} \approx 1.4$, $p_{TA} = \frac{X_{A0}}{K_{TA}} \approx 24.1$, $p_{AF} = \frac{X_{F0}}{K_{FA}} \approx 6.1 \cdot 10^{-4}$, $p_{AT} = \frac{X_{T0}}{K_{TA}} \approx 1.8 \cdot 10^{-4}$, $p_{CF} = \frac{X_{F0}}{K} \approx 6.6$ $p_{CT} = \frac{X_{T0}}{K_{TC}} \approx 1.2 \cdot 10^{-2}$, $p_{CP} = \frac{X_{P0}}{K_{PC}} \approx 0.12$, $p_{AP} = \frac{X_{P0}}{K_{PA}} \approx 3.0 \cdot 10^{-4}$, $p_{PC} = \frac{X_{C0}}{K_{PC}} \approx 14.4$, and $p_{PA} = \frac{X_{A0}}{K_{PA}} \approx 30.1$, with $K = \frac{K_{FC}}{1 + \frac{X_E K_{FC}}{K_{CE} K_{FC^*}}} \approx 30.1$. The numerical values of the dimensionless parameters are calculated using default parameter values listed in section 2.2, which are based on the in the literature found values. Note that only 11 of the 13 parameters are independent, e.g. $p_{CF} = p_{FC} \frac{p_{TA} p_{AF} p_{CT}}{p_{AT} p_{FA} p_{TC}}$ and $p_{CP} = p_{PC} \frac{p_{CF} p_{FA} p_{AP}}{p_{PA} p_{AF} p_{FC}}$. Consequently, we only have 11 free parameters.

In equations 8a to 8e, the following approximation is made: $p_0 = 1 + \frac{X_E}{K_{CE}} + \frac{X_E}{K_{M_{CE}}} = 1 + \frac{X_E}{K_{CE}}(1 + \frac{k_{C^*e}}{k^+_{C^*}}) \approx 1 + \frac{X_E}{K_{CE}}$, since the fraction $\frac{k_{C^*e}}{k^+_{C^*}}$ is assumed to be small ($< 5 \cdot 10^{-5}$, see section 2.2.3). When using the approximation the elastase concentration and the kinetic constants describing the enzyme's activity enters the model solely as the fraction of $X_E$ and $K_{CE}$ ($n_{CE} = \frac{X_E}{K_{CE}}$). We note that some of the other terms may be neglectable in size as well (see section 2.1.3 were a reduced version of the model is stated).

We may numerically solve equations (8a)-(8e) directly given the total concentrations $X_{C0}$, $X_{A0}$, $X_{F0}$, $X_{T0}$, and $X_{P0}$ as input variables and $n_{CE}$ either as a parameter dependent on inflammation status, for known level of elastase as an input variable $n_{CE} = \frac{X_E}{K}$ or for known percentage of CBG on cleaved form ($n_{CC^*}$) by an approximation (see section 2.2.3). Thereby we obtain a solution for the free substances ($X_C, X_F, X_T, X_A, X_P$), and are afterwards able to calculate the concentrations of all complexes using equations (4)-(5g). We will term this model the static model.

*2.1.3. A reduced version of the static model*

As the estimated values of the dimensionless parameters describe, the terms involving $p_{AF}$, $p_{AT}$ and $p_{AP}$ in equation (8d) are much smaller than $x_A$. This results in $x_A \approx 1$, which may be substituted into the equations 8a-8c and 8e. Hence, solving this system of equations is equivalent to solving

$$a_0 x_C^4 + a_1 x_C^3 + a_2 x_C^2 + a_3 x_C + a_4 = 0 \tag{9a}$$

$$x_F = \frac{1}{1 + p_{FA} + p_{FC} x_C} \tag{9b}$$

$$x_T = \frac{1}{1 + p_{TA} + p_{TC} x_C} \tag{9c}$$

$$x_P = \frac{1}{1 + p_{PA} + p_{PC} x_C} \tag{9d}$$

$$x_A = \frac{1}{1 + p_{AF} x_F + p_{AT} x_T + p_{AP} x_P} \tag{9e}$$

in sequential order, where the constant coefficients $a_0$, $a_1$, $a_2$, $a_3$ and $a_4$ are expression in the earlier parameters and can be found in Appendix A. We will term this the reduced static model.

We notice that the present result demands solving a fourth order polynomial equation as is the case in the most recent paper by Nguyen et al. [3] despite the more details of our model such as including elastase, testosterone and progesterone. It should be noted when comparing the two equations that in Nguyen et al. [3] the roots of their fourth order polynomial equation is the free cortisol ($X_F$), while the roots of the fourth order polynomial stated here is the relative variable describing the amount of uncleaved CBG in the free form ($x_C = X_C/X_{C0}$). The relative variable for free cortisol ($x_F = X_F/X_{F0}$) is calculated afterwards as stated in equation 9b.

Since the reduced static model is a fourth order polynomial, an analytical solution or "formula" can be found as was the case of the fourth, third and second order polynomial models of Nguyen et al. [3], Dorin et al. [2], and Coolens et al. [1], respectively. We have stated the only physiological relevant solution in appendix Appendix B. This formula can, as the earlier formulae [3, 2, 1], be calculated in readily accessible programs such as EXCEL[1].

[1] One should be aware of the accuracy of the calculations. Our implementation of

### *2.2. Parameter and input variable values*

#### *2.2.1. Dissociation constants*

As seen in table 1 literature found values of the dissociation constants varies considerably. Some of the variation might be due to difference in methodology. Most of the dissociation constants are determined *in vitro*. According to Dorin et al. [2] their fitting of $K_{FA}$ suggests that the affinity of albumin to cortisol might be higher *in vivo* than what is observed *in vitro* [2].

While the affinity of CBG for cortisol and progesterone varies by temperature, the affinity of human albumin does not, but varies with pH [23]. The affinity of cleaved CBG is about 10-fold reduced compared to native CBG [19, 24].

the solution formulae by Nguyen et al. [3] and Dorin et al. [2] in MATLAB R2016b gave considerably less accurate solutions than solutions found with the inbuilt roots-function. Consequently, in the comparison of the different models' results we use the MATLAB inbuilt roots-function.

Table 1: Equilibrium dissociation constants

| | | |
|---|---|---|
| $K_{FC} = \frac{k^-_{FC}}{k^+_{FC}}$ for Cort:CBG | 0.71 nM | [24]# |
| | 11 nM | [25]# |
| | 13 nM | [7, 26]# |
| | 18 nM | [23] |
| | 19 nM | [27]# |
| | 21 nM | [28]# |
| | 25 nM | [29, 30, 31]# |
| | 32 nM | [19] |
| | **33 nM** | [32] |
| | 39 nM | [33]# |
| | 240 nM | [34] |
| | 292.2 nM | [35] |
| $K_{FC^*} = \frac{k^-_{FC^*}}{k^+_{FC^*}}$ for Cort:CBG$^*$ | 6.25 nM | [24]# |
| | 292 nM | [19] |
| | 1366.0 nM | [35] |
| | **10·K**$_{FC}$ | [3, 19, 24] |
| $K_{FA} = \frac{k^-_{FA}}{k^+_{FA}}$ for Cort:Alb | $1.378 \cdot 10^5$ nM | [2] |
| | $3.00 \cdot 10^5$ nM (at pH 7.8) | [23] |
| | **$3.30 \cdot 10^5$ nM** | [32] |
| | $3.33 \cdot 10^5$ nM | [7]# |
| | $4.796 \cdot 10^5$ nM | [33]# |
| | $8.10 \cdot 10^5$ nM | [36] |
| | $9.00 \cdot 10^5$ nM (at pH 6.8) | [23] |
| $K_{TA} = \frac{k^-_{TA}}{k^+_{TA}}$ for Tes:Alb | $2.46 \cdot 10^4$ nM | [37]# |
| | $2.50 \cdot 10^4$ nM | [7]# |
| | **$2.78 \cdot 10^4$ nM** | [38, 39, 40]# |
| | $2.80 \cdot 10^4$ nM | [27]# |
| | $2.9 \cdot 10^4 - 3.1 \cdot 10^4$ nM | [41]# |
| | $4.00 \cdot 10^4$ nM | [42]# |
| $K_{TC} = \frac{k^-_{TC}}{k^+_{TC}}$ for Tes:CBG | 189 $nM$ | [7]# |
| | **417 nM** | [30]# |
| | 667 $nM$ | [40]# |
| $K_{PC} = \frac{k^-_{PC}}{k^+_{PC}}$ for Prog:CBG | 11.1 $nM$ | [43]# |
| | 38 nM | [31]# |
| | **41.7 nM** | [7, 26]# |
| | 85 nM | [23] |
| $K_{PA} = \frac{k^-_{PA}}{k^+_{PA}}$ for Prog:Alb | **$1.67 \cdot 10^4$ nM** | [7]# |
| | $9.0 \cdot 10^4$ nM (at pH 7.8) | [23] |
| | $20.0 \cdot 10^4$ nM nM (at pH 6.8) | [23] |

Equilibrium dissociation constants with references. Some dissociation constants are calculated from the corresponding association constant marked by a # at the references. Our choice of defeault parameter values are emphasized in bold.

*2.2.2. Steroid and transport protein concentrations*

The reported values for CBG varies considerably (See table S.1). The normal range is wide as reported by Lewis et al. [44] 312 to 1324 nM for 20 normal individuals [44]. Cameron et al. [23] defines three examples of CBG concentration, i.e. high level at 1300 nM, normal at 600 nM, and low at 300 nM [23].

It is possible to measure specifically the uncleaved CBG ($C_{tot}$) with antibodies and afterwards get a relation of how much CBG is elastase cleaved by comparing these measurements with measurements made with other antibodies that binds to both the uncleaved and the elastase cleaved CBG [20] ($C^*_{tot} = X_{C0} - C_{tot}$). According to Lewis and Elder [45] significantly higher levels of CBG measured in both total and intact CBG are seen in women compared to men. The concentration of total and native CBG were $644 \pm 120$ nM and $438 \pm 113$ nM, respectively, in women and $574 \pm 134$ nM and $379 \pm 131$ nM, respectively, for men [45]. For both sexes the ratio of cleaved CBG compared to total CBG, $n_{CC^*} = \frac{C^*_{tot}}{X_{C0}}$, is $0.30 - 0.35$ [45]. For sixteen normal individuals with corresponding levels of total and intact CBG reported in Lewis and Elder [20, figure 6a, p 292] the range of $n_{CC^*}$ is approximately 0 to 0. 635.

The concentration of albumin in the blood is much larger than CBG and the steroids included in the model. The normal range of albumin is $5.49 \cdot 10^5$ nM to $7.20 \cdot 10^5$ nM [46]. However, the level is frequently decreased in elderly people [47].

The normal ranges of testosterone in plasma are 5.83 to 26.30 nM in men and $< 0.35$ to 3.12 nM in women [48]. Lower levels of testosterone are seen in some older compared to younger men ($12.1 \pm 0.7$ nM vs $17.7 \pm 1.0$ nM in Plymate et al. [49]), though the same picture is not visible across all populations [50]. Testosterone concentration normal ranges vary considerably between different laboratories as reported in the resent review by Le et al. [51]. Le et al. [51] attributes some of the variation to the underlying population studies including participants with unknown medical histories [51]. However, also in studies such as Salonia et al. [11], where the participating female subjects were chosen so to be without sexual disorders, the range was wide [11]. Likewise, normal ranges for cortisol varies greatly. Pretorius et al. [4] attributes this not only to differences in the cohorts used to determine the ranges, but also to differences in the techniques used [4]. The assays for determining cortisol concentrations vary greatly [6]. Our findings in the liter-

ature reported in table S.3, S.5, S.6 of the Supplementary materials suggest that the same might hold for progesterone.

The dynamic behaviour of especially cortisol [4], but also testosterone [52, 53, 49] and progesterone [11, 54] should be taken into account. Cortisol levels have great intra-personal variability with a clear circadian rhythm as well as a faster ultradian rhythm with pulses every 1 to 2 hours [55]. The circadian rhythm of cortisol is present in some reference ranges, e.g. Aardal and Holm [56] reports 200 to 800 nM as their serum cortisol reference range at 8 a.m., but $< 300$ nM at 10 p.m. [56]. At 12 p.m. the concentration is even lower $< 50$ nM [57].

It seems that a circadian rhythm in testosterone levels is present in some men [52, 53, 49]. In pregnant women a circadian rhythm has been observed with an inverse relationship to the cortisol rhythm [54, 58]. In women not only progesterone, but also testosterone fluctuates during the menstrual cycle [11].

In men the normal range of progesterone is 0.6 to 4.5 nM, while it for women varies from 0.6 to 4.8 nM in the follicular phase and 5.4 to 85.9 nM in the luteal phase of the menstrual cycle [8]. In pregnant women at term the concentration is approximately 541 nM [12]. CBG [59, 60], cortisol[13, 60], and testosterone concentrations [13] rise during pregnancy, while the concentration of albumin declines [61, 62].

Though the total level of CBG increases in pregnancy, the level of cleaved CBG does not, which results in a smaller ratio of cleaved CBG than normal ($n_{CC^*} = 0.167$) [60]. The increase in CBG during pregnancy is attributed to a direct estrogen-induced rise in production. In women using estrogen-based combined oral contraceptive pills (COCP) the total CBG level is similarly increased [63, 60], but the level of cleaved CBG is also increased, though to a lesser degree ($n_{CC^*} = 0.269$ in COCP women vs $n_{CC^*} = 0.433$ in the normal control women) [60].

### *2.2.3. Elastase activity*

The activity of elastase is described by the input variable or parameter $n_{CE} = \frac{X_E}{K_{CE}}$ with $K_{CE} = \frac{k^+_{C^*}}{k_{C^*_e}} K^{-1}_{M_{CE}}$ as described in section 2.1.2 and 2.1.3.

The level of elastase has been found to be elevated in systemic inflammatory response syndrome (SIRS) patients [64] and in chronic obstructive pulmonary disease (COPD) patients [65]. Higher levels of neutrophil elastase activity have been found in mild and severe preeclampsia [66]. The complex

of neutrophil elastase and the native elastase-inhibitor $\alpha_1$-antitrypsin (NE-$\alpha_1$AT) have in other studies been used as a measure of the release of free elastase and the activity of neutrophils in condition of inflammation [67]. Elevated levels of NE-$\alpha_1$-AT have been found in e.g. patients with Crohns disease (CD), ulcerative colitis (UC) [68], food hypersensitivity [69], and intermediate uveitis (IU) [67].

The magnitude of the increase in elastase activity differs between diseases and reported studies from a less than 2 fold increase in [66], 2-3 fold increase in Polańska et al. [67] and Pawlica-Gosiewska et al. [68], 4 fold increase in Zbikowska-Gotz et al. [69] and up to more than 10 fold increase in Kodama et al. [64]. In 20 normal subjects Donnelly et al. [70] found a mean of 0.631 nM and a range of 0.31 to 1.73 nM elastase [70]. In multiple trauma patients the concentration ranged from 0.725 to 23.051 nM [70].

The elimination constant $k_{C^*e}$ for human CBG$^*$ is reported to be the same as for uncleaved CBG in a study done in rabbits [17]. In humans the half life of CBG is approximately 5 days (range measured 4.6 to 6.0 days in five subjects) [71], i.e. $k_{C^*e} = k_{Ce} \approx 1 \cdot 10^{-4}$ min$^{-1}$.

It has been difficult to find measurements of $K_{M_{CE}}$ and $k^+_{C^*}$ in the literature. For other substrates in the literature reported values of the Michaelis-Menten constant of human neutrophil elastase range from $1 \cdot 10^3$ to $3.7 \cdot 10^6$ nM and the catalytic constant ($k_{cat}$) from 2.4 to 288000 min$^{-1}$[72]. As an example the $K_M = 1.4 \cdot 10^5 nM$ and the catalytic constant $k_{cat} = 1.0 \cdot 10^3$ min$^{-1}$ for elastase's cleavage of methoxysuccinyl-Ala-Ala-Pro-Val-4-nitroanilide [73]. However, CBG as opposed to methoxysuccinyl-Ala-Ala-Pro-Val-4-nitroanilide and a lot of other elastase substrates after cleavage does not inhibit the further activity of elastase and Hammond et al. [74] describe the cleavage of CBG by elastase as extremely efficient [74] and Sumer-Bayraktar et al. [75] the reaction as fast.

For unknown elastase level, but known $C_{tot}$ and $C^*_{tot}$, we will make an approximated input variable expression. From equation 4, we have that $\frac{X_E}{K_{CE}}$ is equivalent to the *a priori* unknown fraction of $X_{C^*}$ to $X_C$. However, leaving out the progesterone, testosterone and elastase bound fractions we approximately have $C_{tot} \approx X_C(1 + \frac{X_F}{K_{FC}})$ and $C^*_{tot} \approx X_C \frac{X_E}{K_{CE}}(1 + \frac{X_F}{K_{FC^*}})$. Looking at the fraction of $C^*_{tot}$ to $C_{tot}$ and isolating $\frac{X_E}{K_{CE}}$ we get:

$$\frac{X_E}{K_{CE}} = \frac{(1 + \frac{X_F}{K_{FC}})}{(1 + \frac{X_F}{K_{FC^*}})} \frac{C^*_{tot}}{C_{tot}} = k_{nce} \frac{C^*_{tot}}{C_{tot}} = k_{nce} \frac{n_{CC^*}}{1 - n_{CC^*}} \tag{10}$$

The factor $k_{nce} = \frac{(1+\frac{X_F}{K_{FC}})}{(1+\frac{X_F}{K_{FC^*}})}$ is not a true constant, since it depends on the level of free cortisol, $X_F$. The range of $X_F$ in normal subjects is 5.5 to 38.9 nM in the morning [44], while higher levels can be found in e.g. sepsis [76] (for more values found in the literature see table S.3 in Supplementary Materials). Default values for the dissociation constants are $K_{FC}$=33 nM and $K_{FC^*} = 10 \cdot K_{FC}$ (see table 1). This being so, we may use $k_{nce} = 2$ as a gross estimate.

*2.2.4. Default parameter and input-variable values*

In section 3 the sensitivities and performances of the static model (see section 2.1.2) and the reduced static model (see section 2.1.3) are compared to the performances of the models presented in Coolens et al. [1], Dorin et al. [2], Nguyen et al. [3].

The input variable $X_{C0}$ and $X_{F0}$ as well as the parameter $K_{FC}$ (in Coolens et al. [1] as the association constant, $\frac{1}{K_{FC}}$) are present in all five models. $X_{A0}$ and $K_{FA}$ are included in all models except Coolens et al. [1].

Nguyen et al. [3] and our models include $K_{FC^*}$. A parameter $n_{CC^*} = \frac{{C^*}_{tot}}{X_{C0}}$ describes the percentage of CBG on cleaved form. The total concentrations of cleaved and uncleaved CBG (${C^*}_{tot}$ and $C_{tot}$, respectively) are only explicitly present in the model by Nguyen et al. [3], but with the approximation $n_{CE} \approx \frac{k_{ne}{C^*}_{tot}}{C_{tot}} = \frac{k_{ne} \cdot n_{CC^*}}{1-n_{CC^*}}$ enters our model as well. However, with our model one can chose to explicitly include the activity of elastase measured as $n_{CE} = \frac{X_E}{K_{CE}}$. Additionally, our models include $X_{T0}$ and $X_{P0}$ as well as $K_{TC}$, $K_{PC}$, $K_{TA}$, and $K_{PA}$.

The chosen default values of the equilibrium dissociation constants are emphasised in bold in table 1 and the input variables in table 2. For some analysis, including the sensitivity analysis, the total concentrations of testosterone and progesterone are set to the maximums of their normal ranges in order to investigate the maximal effect, i.e. $X_{T0} = 26.30$ nM (men [48]), and $X_{P0} = 85.9\ nM$ (women in luteal phase [8]). We use the estimate $n_{CE} = \frac{X_E}{K_{CE}} \approx k_{nce}\frac{n_{CC^*}}{1-n_{CC^*}}$ when *a priori* values for the concentrations of CBG and CBG$^*$ are known from measurements and the default $k_{nce} = 2$. When not known, the default ratio of cleaved CBG to total CBG is set to $n_{CC^*} = 0.325$.

These default values are in reasonable agreement with the dissociation constants listed in table 1 in section 2.2.1, the normal ranges described in section 2.2.2, and the additional values and normal ranges listed in table S.1

Table 2: Default input variable values as well as representative population groups

| | Default | YM | OM | YWL | YWF | PW3T | COCP |
|---|---|---|---|---|---|---|---|
| $X_{C0}$ [nM] | **600** | 574 | 574 | 644 | 644 | 877 | 1093 |
| $X_{A0}$ [$\cdot 10^4$nM] | **67** | 70.1 | 66.2 | 70.1 | 70.1 | 58.4 | 70.1 |
| $X_{F0}$ [nM] | **200** | 210 | 210 | 210 | 210 | 630 | 630 |
| $X_{P0}$ [nM] | **5 (85.9)** | 1.5 | 1.5 | 85.9 | 1.5 | 575 | 0.86 |
| $X_{T0}$ [nM] | **5 (26.3)** | 26.3 | 12.1 | 1.4 | 1.4 | 2.8 | 1.4 |
| $n_{CC^*}$ [ ] | **0.325** | 0.343 | 0.343 | 0.343 | 0.343 | 0.167 | 0.269 |
| $n_{CE} \approx \frac{2 \cdot n_{CC^*}}{1-n_{CC^*}}$ [] | **0.963** | 1.044 | 1.044 | 1.044 | 1.044 | 0.401 | 0.736 |

Default input variable values are shown in bold. For some analysis of $X_{P0}$ and $X_{T0}$ the values stated in the brackets corresponding to the maximum in the normal range for young women and men are used. Additionally, estimated 24h mean input variable values in different population groups are shown; YM = young men, OM= old men, YWL= young women in the luteal phase, YWF= young women in the folicular phase, PW3T = pregnant women in the 3rd trimester, and COCP=women tacking estrogen-based combined oral contraceptive pills. The values are chosen by the relations described in section 2.2.2 and from values found in [77, 63, 45, 60, 61, 78, 58, 13, 10, 12, 11, 79, 49, 14, 80, 48].

to S.6 in Supplementary materials.

In section 3.2 we investigate the effect of individually varying the parameters and the input variables in the normal range reported in the literature. In 3.4 the combined effect of varying the input variables as typically seen in individuals of different age and gender is shown with estimated input variable values for a typical young man (YM), old man (OM), young woman in the luteal phase (YWL), young woman in the folicular phase (YWF), pregnant woman in the third trimester (PW3T), and a woman using estrogen-based combined oral contraceptive pills (COCP) (see table 2).

### *2.3. Data of four specific subjects*

In Lewis and Elder [20] data of total CBG, native CBG and free cortisol levels measured by ultrafiltration/ligand binding can be found for four patients (see Lewis and Elder [20, table 1, p.293]). Two of these patients were termed discordant (sample Discordant 1 and 2), since the levels of intact CBG were less than 50% of total plasma CBG levels. For the rest of the samples that Lewis and Elder [20] looked at, the differences between total and intact CBG levels were less than 20%. Hence, these samples were termed concordant and the data of two of these (sample Concordant 1 and 2) were shown in the same table as the discordant data for comparison [20].

Nguyen et al. [3] used the data of the four patients found in Lewis and Elder [20] to compare the predictability of Coolens [1], the cubic [2] and their own formulae. Since the albumin concentrations for these samples are not measured, Nguyen et al. [3] made two cases for albumin affinity with $K_{FA}$=330,000 nM (case A) and $K_{FA} = 137,800nM$ (case B). Using the ratio of total albumin to its dissociation constant $N = X_{A0}/K_{FA} = 1.74$ of Coolens et al. [1], the total albumin concentrations of case A and B are 574,200 nM and 239,772 nM, respectively. Our models predictions for these two cases are tested with $n_{CE}$ approximated by $n_{CE} \approx \frac{k_{nce}C^*_{tot}}{C_{tot}}$ with the default $k_{nce} = 2$.

In a third case C with albumin affinity and concentration as in case A, the activity of neutrophil elastase $n_{CE}$ is varied individually for each of the four subjects. In a fourth case D, the parameter $k_{nce}$ is fitted as one value common for the four subjects, but different from the default value $k_{nce} = 2$.

### *2.4. Method of sensitivity analysis*

The change in concentration of free cortisol as a result of a $\pm 1\%$ variation from the default value of a given parameter $\Theta_i$ and normalised to the change in the parameter value is used as a measure of absolute sensitivity ($s_a(\Theta_{i1\%}) = \frac{\Delta X_F(\Theta_{i\pm 1\%})}{\Delta \Theta_{i\pm 1\%}} = \frac{X_F(1.01\Theta_{i_{default}}) - X_F(0.99\Theta_{i_{default}})}{0.02\Theta_{i_{default}}}$). The sensitivity analysis is local, since it depends on the parameter values chosen as default values.

We will investigate the sensitivity of our models for both $n_{CC^*}$ and $n_{CE}$ by using the $n_{CC^*}$-dependent approximation of $n_{CE}$ for all runs except for the one where the sensitivity towards $n_{CE}$ is investigated.

The relative sensitivities is considered as well ($s_r(\Theta_{i1\%}) = \frac{\Theta_{i_{default}}}{X_F(\Theta_{i_{default}})} \cdot \frac{\Delta X_F(\Theta_{i\pm 1\%})}{\Delta \Theta_{i\pm 1\%}}$), since the sizes of the parameters vary greatly compared to each other (see section 2.2.4).

Furthermore, the differences in variation of the individual parameters are considerable (see section 2.2.1 to 2.2.4). Hence, we define a measure, where the sensitivities are normalised by a factor ($\Delta_{literature}$) determined by the variation of the parameters seen in the literature ($s_l(\Theta_{i1\%}) = \Delta_{literature} \frac{\Delta X_F(\Theta_{i\pm 1\%})}{\Delta \Theta_{i\pm 1\%}}$). For the dissociation constants the differences between the largest and smallest values reported are used as $\Delta_{literature}$, while the differences between the upper and lower limits of the normal ranges with both genders considered are used for the input variables, which gives $\Delta_{literature}(X_{C0}) = 1012$ nM, $\Delta_{literature}(X_{F0}) = 750$ nM, $\Delta_{literature}(X_{A0}) = 1.71e5$ nM, $\Delta_{literature}(n_{CC^*}) =$

0.635, $\Delta_{literature}(n_{CE})$, $\Delta_{literature}(X_{T0}) = 26.3$ nM, $\Delta_{literature}(X_{P0}) = 85.9$ nM, $\Delta_{literature}(K_{FC}) = \Delta_{literature}(K_{FC^*}) = 291$ nM, $\Delta_{literature}(K_{FA}) = 7.62e5$ nM, $\Delta_{literature}(K_{TC}) = 478$ nM, $\Delta_{literature}(K_{TA} = 1.54e4$ nM, $\Delta_{literature}(K_{PC}) = 73.9$ nM, and $\Delta_{literature}(K_{PA}) = 18.33e4$ nM.

## 3. Results

### *3.1. Sensitivity analysis*

All five models show an across models similar sensitivity towards the parameters included in multiple models, e.g. $s_a(K_{FC})$ is 0.342, 0.356, 0.283, and 0.296 for our static model, Nguyen et al. [3], Dorin et al. [2], and Coolens et al. [1], respectively (see figure 1). The reduced static model shows close to identical sensitivities to the static model. Ranking the absolute values of the absolute sensitivities ($s_a$) for the parameters of our static model, the ascending order of the parameters is: $K_{TA}$, $X_{A0}$, $K_{FA}$, $K_{PA}$, $K_{TC}$, $X_{T0}$, $X_{P0}$, $K_{FC^*}$, $K_{PC}$, $X_{C0}$, $X_{F0}$, $K_{FC}$, $n_{CE}$, $n_{CC^*}$. The same sequence applies to sensitivities of the other models towards common parameters.

If we divide the ranked absolute sensitivities into four categories; our static model shows low sensitivity towards $K_{TA}$, $X_{A0}$, $K_{FA}$, $K_{PA}$, $K_{TC}$, and $X_{T0}$ ($5.13 \cdot 10^{-7}$ to $5.84 \cdot 10^{-4}$), is sensitive towards $X_{P0}$, $K_{FC^*}$, and $K_{PC}$ ($3.19 \cdot 10^{-3}$ to $5.78 \cdot 10^{-3}$), very sensitive towards $X_{C0}$, $X_{F0}$, $K_{FC}$ ($2.71 \cdot 10^{-2}$ to $3.42 \cdot 10^{-1}$), and extremely sensitive towards $n_{CE}$ and $n_{CC^*}$ (5.05 to 22.15).

As seen by the sensitivity for the parameter $n_{CE}$, our model is sensitive towards including elastase. The seemingly greater sensitivity towards changing $n_{CC^*}$ is due to the approximation $n_{CE} = \frac{X_E}{K_{CE}} \approx \frac{2 \cdot n_{CC^*}}{1 - n_{CC^*}}$ (see section 2.4). Hence, a percentage change of $n_{CC^*}$ gives a larger percentage change than changing $n_{CE}$ directly by the same percentage. The approximation will be investigated further in section 3.2.4 and 3.3.

All five models are very sensitive towards the three input variables and parameters included in the original model by Coolens et al. [1], i.e. native CBG-cortisol dissociation constant ($K_{FC}$), the total concentration of CBG ($X_{C0}$), and the total concentrations of cortisol ($X_{F0}$).

Looking at the parameters describing the influence of progesterone and testosterone ($K_{TA}$, $K_{PA}$, $K_{TC}$, $X_{T0}$, $X_{P0}$, $K_{PC}$), which are unique for our static model compared to the models by Coolens et al. [1], Dorin et al. [2], Nguyen et al. [3], our static model have similar sensitivities towards $X_{P0}$ and $K_{PC}$ as towards the dissociation constant of cortisol and cleaved CBG

($K_{FC^*}$). The sensitivities towards the testosterone parameters ($K_{TA}$, $K_{TC}$, $X_{T0}$) are all low ($< 10^{-3}$).

The models by Dorin et al. [2], Nguyen et al. [3] and ours all include an input variable $X_{A0}$ describing the concentration of albumin and the dissociation constant $K_{FA}$. In our model parameters describing the binding of progesterone and testosterone are included as well. Interestingly, all of the parameters describing albumin's interaction with the steroids fall in the low absolute sensitivity category. If we take into account the size difference of the parameters by looking at relative sensitivities ($s_r(\Theta_{i1\%})$), the relative sensitivities for albumin's influence are similar to the relative sensitivities towards the parameters describing CBG on different forms (for the static model the ascending order is $K_{TA}$, $K_{TC}$, $X_{T0}$, $K_{PA}$, $K_{PC}$, $X_{P0}$, $K_{FC^*}$, $K_{FA}$, $X_{A0}$, $n_{CE}$, $n_{CC^*}$, $K_{FC}$, $X_{C0}$, $X_{F0}$, see figure S.1a in Supplementary materials). However, when we take into account the inter- and intra-individual variations in the parameters and input variables by looking at the sensitivities related to the variance of the parameters seen in the literature ($s_l(\Theta_{i1\%})$), the importance of varying the size of $X_{A0}$ in the normal range seems to be small (for the static model the ascending order is $K_{TA}$, $X_{T0}$, $K_{TC}$, $X_{P0}$, $K_{PC}$, $K_{FC^*}$, $X_{A0}$, $K_{PA}$, $K_{FA}$, $n_{CC^*}$, $n_{CE}$, $X_{C0}$, $X_{F0}$, $K_{FC}$ , see figure S.1b in Supplementary materials).

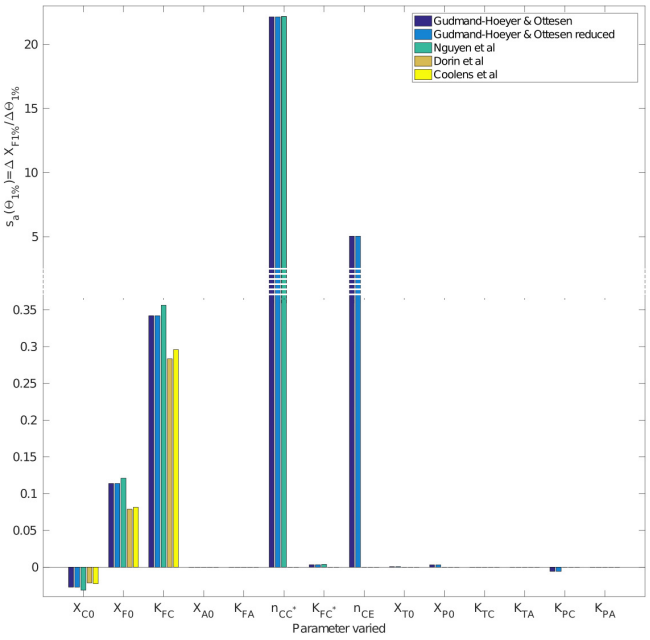



Figure 1: The absolute sensitivities towards common parameters are similar across the five models. Absolute sensitivities of the static model (Gudmand-Hoeyer & Ottesen) and reduced static model (Gudmand-Hoeyer & Ottesen reduced) compared to the sensitivities of the models by Nguyen et al. [3], Dorin et al. [2], and Coolens et al. [1] to a 1% variation in the parameter values ($s_a(\Theta_{i1\%})$). Change in scale of $s_a(\Theta_{i1\%})$ is indicated as dotted axis and bars.

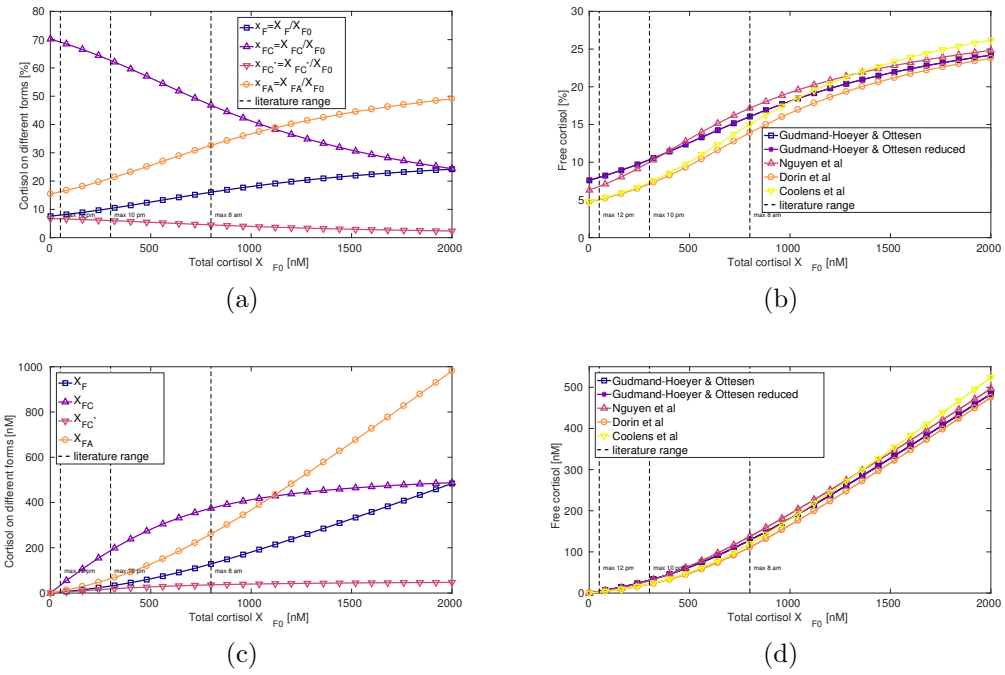
### *3.2. Individual variation of parameters and input variable values*

#### *3.2.1. Variation in total cortisol concentrations*

The influence of varying total cortisol ($X_{F0}$) in the range of 0 to 2000 nM on calculated measures of free cortisol is shown in figure 2. In figure 2a the calculated cortisol on different forms for the static model is shown, while calculated free cortisol for the static model and the reduced static model as well as the models in Nguyen et al. [3], Dorin et al. [2], and Coolens et al. [1] are shown in figure 2b for comparisons. Figure 2c and 2d show the results in absolute concentrations [nM]. The results of the static model and the reduced static model are close to identical (see figure 2b and 2d). All parameters and input variables beside $X_{F0}$ are kept as their default values (see section 2.2.4).

All models in figure 2b give a similar sigmoid relation in calculated free cortisol percentage ($x_F$) for the variation of $X_{F0}$, though with different maximum value, slope and turning point. The model by Coolens et al. [1] starts at the lowest free cortisol percentage, but end up with the highest value. The results of the static and the reduced static model is practically identical.

The circadian rhythm of the cortisol level is indicated on the figure by depicting maximum levels for 8 a.m., 10 p.m. and 12 p.m. normal ranges (800 nM, 300 nM [56] and 50 nM [57], respectively). Though some circadian variation can be present in some of the other input variables (CBG, progesterone and testosterone), cortisol is by far the substance with greatest intra-personal variation. Hence, figure 2 shows that there is a great variation of the level of free cortisol both measured in percentage and in absolute concentration during a normal day. Figure 2c and 2d show that the actual changes measured in nM are large and that the CBG-bound fractions ($X_{FC}$ and $X_{FC^*}$) both get close to saturation levels in the cortisol peak hours of the day (8 a.m. max level). Meanwhile the fraction of cortisol bound to albumin ($X_{FA}$) still increases at $X_{F0} = 2000$ nM.




(a) (b) (c) (d)

Figure 2: The static, the reduced static and the models of [1], [2], and [3] show qualitatively similar responses to change in total cortisol, but difference in actual concentrations. The total concentration of cortisol ($X_{F0}$) is varied. Maximum $X_{F0}$ levels for 12 p.m., 10 p.m., and 8 a.m. normal ranges are indicated by vertical dotted lines (50 nM [57], 300 nM, 800 nM [56], respectively). a) The calculated concentrations of cortisol in all forms as percentage of total cortisol given is shown for the static model (Gudmand-Hoeyer & Ottesen). b) The calculated concentrations of cortisol in the free form for the static model (Gudmand-Hoeyer & Ottesen) and the reduced static model (Gudmand-Hoeyer & Ottesen reduced) as well as the models in Nguyen et al. [3] (Nguyen et al), Coolens et al. [1] (Coolens et al), and Dorin et al. [2] (Dorin et al) are shown as percentages of total cortisol. c) As 2a, but in absolute concentrations [nM]. d) As 2b, but in absolute concentrations [nM]. The reduced static and the static model show close to identical results.

*3.2.2. The impact of albumin variation*

In figure 3 the total albumin concentration ($X_{A0}$) and the albumin-cortisol dissociation constant ($K_{FA}$), respectively, are varied.

As seen on figure 2a and even clearer on figure 2c the concentration of albumin-bound cortisol ($X_{FA}$) is far from saturated, when cortisol is varied 0 to 2000 nM for the default parameter values. Nguyen et al. [3] refer to the relationship between albumin and cortisol as non-saturable due to the high concentration of albumin and large dissociation constant [3]. As the ratio of the affinity constant and the concentration of albumin is assumed constant in the model of Coolens et al. [1], changing $X_{A0}$ or $K_{FA}$ do not influence the result of their model (see figure 3b and 3d). The effect of changing $X_{A0}$ or $K_{FA}$ on the prediction of the model by Nguyen et al. [3] is similar to ours though the absolute values are different, while the effect on the predictions of Dorin et al. [2] is smaller.

The sensitivity analysis showed a very low sensitivity of the static model towards all parameters and input variable related to albumin ($X_{A0}$, $K_{FA}$, $K_{PA}$, $K_{TA}$). However, this does not mean that albumin is unimportant in the calculation of free cortisol. The sensitivity analysis was preformed with free cortisol as the model output. The decrease in free cortisol percentage is small (0.56 percentage points), when total albumin ($X_{A0}$) is varied from the minimum value $5.49 \cdot 10^5$ nM to the maximum value $7.20 \cdot 10^5$ nM of the normal range reported in Rustad et al. [46] (see figure 3) in good keeping with the sensitivity analysis. However, when looking at the distribution of cortisol on different forms in figure 3a cortisol bound to albumin simultaneously increases by 3.81 percentage points and cortisol bound to CBG on different forms decreases by 3.25 percentage points. Increasing the affinity of albumin for cortisol by varying $K_{FA}$ from the maximum to the minimum of the in the literature reported values have a larger effect by decreasing cortisol in the free form 3.81 percentage points, while CBG-bound forms decrease by 22.70 percentage points and albumin bound increase by 26.51 percentage points.

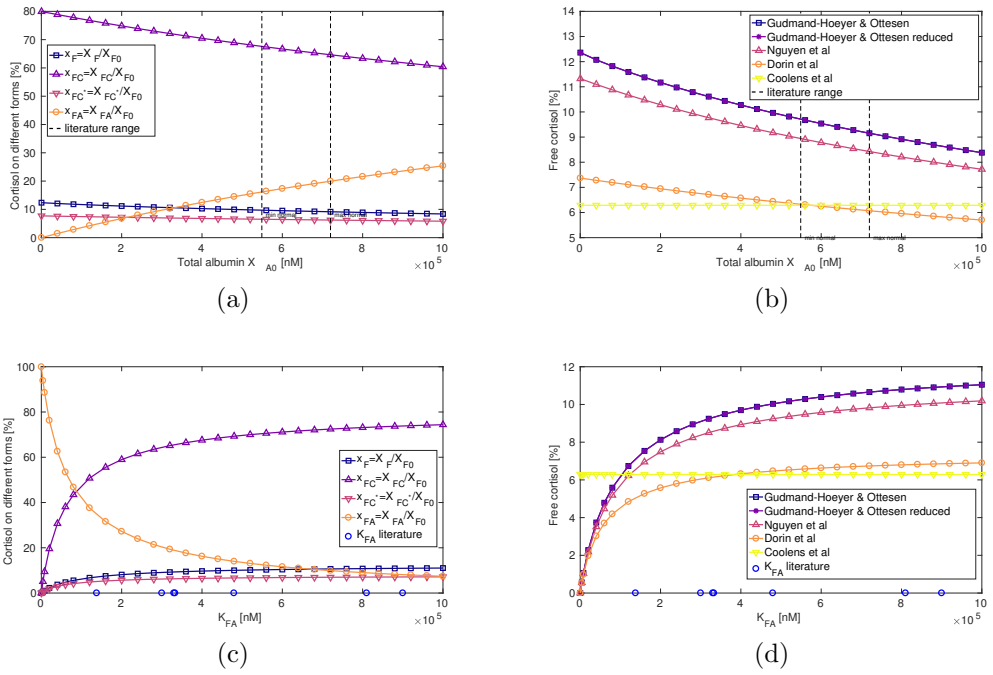


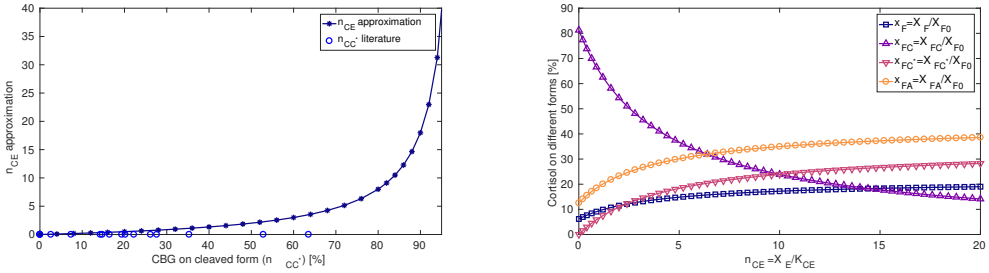

Figure 3: Varying total albumin and its dissociation constant for cortisol have great impact on redistribution of cortisol on bound forms. The total concentration of albumin ($X_{A0}$) is varied in a) and b) and the dissociation constant of albumin for cortisol ($K_{FA}$) in c) and d). Minimum and maximum values for the normal range of albumin is indicated by vertical dotted lines ($5.49 \cdot 10^5$ nM to $7.20 \cdot 10^5$ nM [46]). The $K_{FA}$ values found in the literature (see table 1) is marked with circles on the x-axis. In a) and c) the calculated concentrations of cortisol in all forms as percentage of total cortisol is shown for the static model. In b) and d) the calculated concentrations of cortisol in the free form for the static model (Gudmand-Hoeyer & Ottesen) and the reduced static model (Gudmand-Hoeyer & Ottesen reduced) as well as the models in Nguyen et al. [3], Coolens et al. [1], and Dorin et al. [2] are shown as percentages of total cortisol. The reduced static and the static model show close to identical results.

### *3.2.3. The impact of CBG variation*

As the sensitivity analysis in section 3.1 indicated, varying $X_{C0}$ has a large impact on the level of free cortisol. A wide normal range such as the one reported in Lewis et al. [44] of 312 to 1324 nM gives a 10.94 percentage points difference in by the static model estimated free cortisol percentage between maximum and minimum normal range values (see figure S.4 in Supplementary materials). Hence, the percentage of free cortisol could vary greatly from person to person and with the diurnal rhythm of CBG and cortisol combined in one person as well.

Common for all the models compared the percentage of free cortisol varies greatly between the smallest and the largest $K_{FC}$ reported in the literature, though the exact values are different with Nguyen et al. [3] and ours versus Coolens et al. [1] and Dorin et al. [2] being close in their predictions. The prediction for free cortisol for the static model changes 24.21 percentage points in this interval. The redistribution of cortisol bound to CBG or to albumin is even larger (see figure S.4 in Supplementary materials).

### *3.2.4. Variation in the activity of elastase*




(a) (b)

Figure 4: The cleavage of CBG by elastase has a great impact on cortisol distribution. a) The approximation of $n_{CE}$ as a function of how much of CBG is on cleaved form ( $n_{CE} = \frac{X_E}{K_{CE}} \approx \frac{k_{nce} \cdot n_{CC^*}}{1-n_{CC^*}}$, with $k_{nce} = 2$ ). On the x-axis $n_{CC^*}$-values for 16 normal subjects from Lewis and Elder [20] are indicated with circles. b) The fraction $n_{CE} = \frac{X_E}{K_{CE}}$ is varied. The calculated concentrations of cortisol in all forms as percentage of total cortisol given is shown for the static model.

Changing the fraction of CBG on elastase-cleaved form ($n_{CC^*} = \frac{CBG^*_{tot}}{X_{C0}}$) influence the model by Nguyen et al. [3] and through the approximation $n_{CE} \approx \frac{2 \cdot n_{CC^*}}{1-n_{CC^*}}$ the static and the reduced static model in a very similar way.

The prediction by Coolens et al. [1] and Dorin et al. [2] are unaffected by change in $n_{CC^*}$, since the differentiation between CBG on cleaved and uncleaved form is not included in their models (see figure S.7 in Supplementary materials).

In figure 4a the approximation of $n_{CE}$ as a function of $n_{CC^*}$ is shown in the interval 0 to 95% to illustrate what happened to the approximated $n_{CE}$, when $n_{CC^*}$ is changed. The $n_{CC^*}$ for 16 normal subjects reported in Lewis and Elder [20] is indicated as circles on the x-axis. The approximated $n_{CE}$ values are similar for all 16 subjects including the two discordant samples compared to the drastic rise in approximated $n_{CE}$ values for higher $n_{CC^*}$ values.

In figure 4b the parameter $n_{CE}$ is varied from 0 to 20. As the figure shows, both models presented in this paper indicate that free cortisol concentration is very sensitive towards change in elastase activity.

The default value for the parameter $n_{CC^*}$ set to 0.325 gives with the approximation a default value of 0.963 for $n_{CE}$. In section 3.3 we investigate what happens, when we fit $n_{CE}$ individually to the data of four individuals with known levels of total cortisol, free cortisol, native CBG and cleaved CBG.

*3.2.5. The impact of testosterone and progesterone variation*

The steroid hormones testosterone and progesterone are included in our model as opposed to the three earlier models [1, 2, 3]. Hence, we are able to investigate their influences on free cortisol concentrations by varying the total concentrations $X_{T0}$ and $X_{P0}$ as well as their dissociation constants $K_{TC}$, $K_{TA}$, $K_{PC}$, and $K_{PA}$.

The effect of varying testosterone in the normal range for young men (max 26.3 nM [48]) has an impact of $< 0.03$ percentage points on cortisol on any form. Setting $X_{T0} = 26.3nM$ the effect of varying $K_{TC}$ and $K_{TA}$ in the range of the in the literature found values are $< 0.01$ percentage points in all forms of cortisol.

The effects of changing $K_{PC}$ and $K_{PA}$ are small ($< 0.1$ percentage points change of cortisol on any form, see figure S.10), when progesterone levels are at 85.9 nM or less, but could have a higher impact for greater progesterone levels.

Figure 5 indicates that the fluctuations in progesterone levels seen during a normal menstrual cycle could affect free cortisol levels. A progesterone level of 85.9 nM, which is the maximum of the normal range in the luteal phase,

increases the predicted free cortisol level by 0.14 percentage points. When the progesterone concentration increases some of the cortisol no longer bound to CBG binds to albumin instead (see figure 5). Including progesterone at the level seen in late pregnancy have a similar impact on cortisol distribution as changing albumin concentrations in the normal range, since free cortisol is increased by 0.85 percentage points, the fraction bound to albumin is increased by 1.70 percentage points, while the fraction bound to CBG is decreased by 2.55 percentage points.

However, these calculations are done by only changing the progesterone levels, while keeping all other parameters and input variables as their default values. In section 3.4 we will look at the predictions of the compared models on typical levels of all input variables for different population groups with pregnant women as one of these.

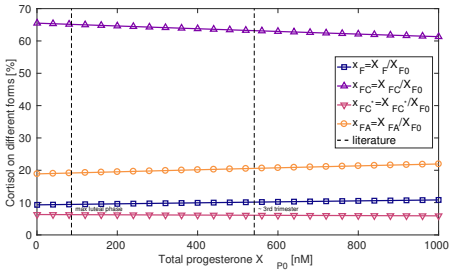



Figure 5: Progesterone at levels seen in the luteal phase and during pregnancy could influence the cortisol distribution. The total concentration of progesterone ($X_{P0}$) is varied. The maximum of the luteal phase (85.9 nM [8]) and the normal level of 3th trimester pregnancy (541 nM [12]) are marked with vertical dotted lines. The calculated concentrations of cortisol in all forms as percentage of total cortisol are shown for the static model.

### *3.3. Different models' predictions for four individuals with measured free cortisol*

In table 3 estimates of free cortisol for four subjects described in [20] and [3] by the different methods stated in section 2.3 appear. The results are shown graphically in a bar diagram in figure 6.

The approximated values for $\frac{X_E}{K_{CE}} \approx \frac{2C^*_{tot}}{C_{tot}}$ in case A and B are 0.49, 0.32, 2.18, and 3.99 for subject Concordant 1, Concordant 2, Discordant 1, and Discordant 2, respectively, while the corresponding individually fitted values in case C are 0.44, 0.10, 0.93, and 0.84, respectively. Comparing the individually fitted $n_{CE}$ values with the approximated $\frac{X_E}{K_{CE}} \approx \frac{2C^*_{tot}}{C_{tot}}$ for the

four individual subjects, one see that for the concordant subjects the values are close, while they differ more for the discordant subjects.

With the individually fitted values for $n_{CE}$ in case C the model predictions are really close to data (see table 3 and figure 6). In case D $n_{CE}$ is approximated from the level of cleaved CBG by $n_{CE} \approx \frac{k_{nce} \cdot n_{CC^*}}{1-n_{CC^*}}$ and the factor $k_{nce}$ is fitted as one common factor for all four subjects. The fitted value is $k_{nce} = 0.63$, i.e. the $n_{CE}$-values are 0.15, 0.10, 0.69, and 1.26 for subject Concordant 1, Concordant 2, Discordant 1, and Discordant 2, respectively. The fit of our model in case D is not as perfect as with the individual fitted $n_{CE}$ in case C. However, our model with this common factor still performs better than any of the other models for three out of four subjects.

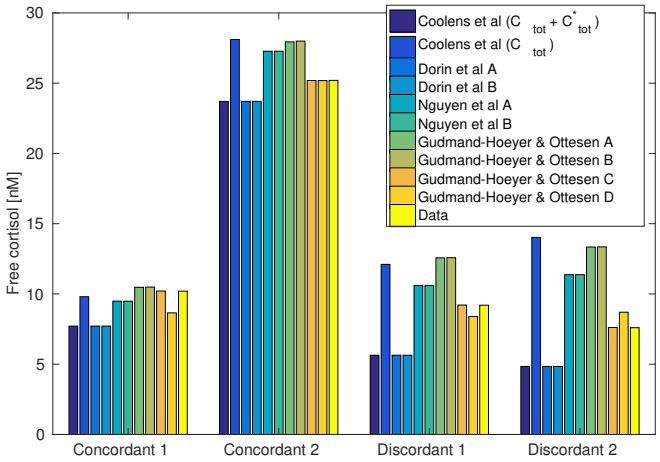



Figure 6: The model fits data excellently compared to previous models, when the elastase activity is fitted. Model predictions by Coolens et al. [1], Dorin et al. [2], Nguyen et al. [3] and our static model (Gudmand-Hoeyer & Ottesen's formula) and data of free cortisol compared graphically for each of the four subjects Concordant 1, Concordant 2, Discordant 1, and Discordant 2 described in [20]. Letters A, B, C, and D refer to the corresponding cases described in section 2.3. Measured input variables of [20] can be found in table 3. The brackets after Coolens refer to whether the results were calculated on total CBG ($C_{tot} + C^*_{tot}$) or only intact CBG ($C_{tot}$).

Table 3: Measured and estimated free cortisol of four subjects described in [20].

| Sample | Concordant 1 | Concordant 2 | Discordant 1 | Discordant 2 |
|---|---|---|---|---|
| $C_{tot}$ (nM) | 830 | 500 | 480 | 297 |
| $C^*{}_{tot}$ (nM) | 204 | 79 | 524 | 592 |
| $X_{F0}$ | 217 | 307 | 162 | 127 |
| $X_F$ (nM) | **10.2** | **25.2** | **9.2** | **7.6** |
| $X_F$ (%) | 4.70 | 8.21 | 5.68 | 5.98 |
| Coolens' formula: | | | | |
| On $C_{tot} + C^*{}_{tot}$ | 7.7 | 23.7 | 5.6 | 4.8 |
| On $C_{tot}$ | 9.8 | 28.1 | 12.1 | 14.0 |
| Dorin's formula: | | | | |
| Case A | 7.7 | 23.7 | 5.6 | 4.8 |
| Case B | 7.7 | 23.7 | 5.6 | 4.8 |
| Nguyen's formula | | | | |
| Case A | 9.5 | 27.3 | 10.6 | 11.4 |
| Case B | 9.5 | 27.3 | 10.6 | 11.4 |
| Gudmand-Hoeyer & Ottesen's formula | | | | |
| Case A | 10.5 | 27.9 | 12.6 | 13.3 |
| Case B | 10.5 | 28.0 | 12.6 | 13.4 |
| Case C | 10.2 | 25.2 | 9.2 | 7.6 |
| Case D | 8.7 | 25.2 | 8.4 | 8.7 |

Measured native CBG concentration ($C_{tot}$), cleaved CBG$^*$ concentration ($C^*{}_{tot}$) and total and free cortisol concentrations ($X_{F0}$ and $X_F$) are shown for samples from four subjects Concordant 1, Concordant 2, Discordant 1, and Discordant 2 described in [20]. The results of different methods for calculating free cortisol are reported: Coolens et al. [1], Dorin et al. [2], Nguyen et al. [3] and our static model (Gudmand-Hoeyer & Ottesen's formula). See a description of case A to D in section 2.3.

### *3.4. The impact of gender and age differences in albumin, testosterone and progesterone*

Figure 7 shows the predicted distribution of cortisol in blood for estimated 24h mean input variable values in different population groups made with the models by Coolens et al. [1] on total CBG (Coolens ($C_{tot} + C^*_{tot}$)), Coolens et al. [1] only including uncleaved CBG (Coolens ($C_{tot}$)), Dorin et al. [2] (Dorin), Nguyen et al. [3] (Nguyen), our static model without the influence of progesterone and testosterone (G & O w/o P,T), our static model (G & O), and the reduced static model (G & O reduced) for estimated 24h averages of albumin, CBG, CBG$^*$, cortisol, progesterone and testosterone. The population groups are young men (7a), old men (7b), young women in

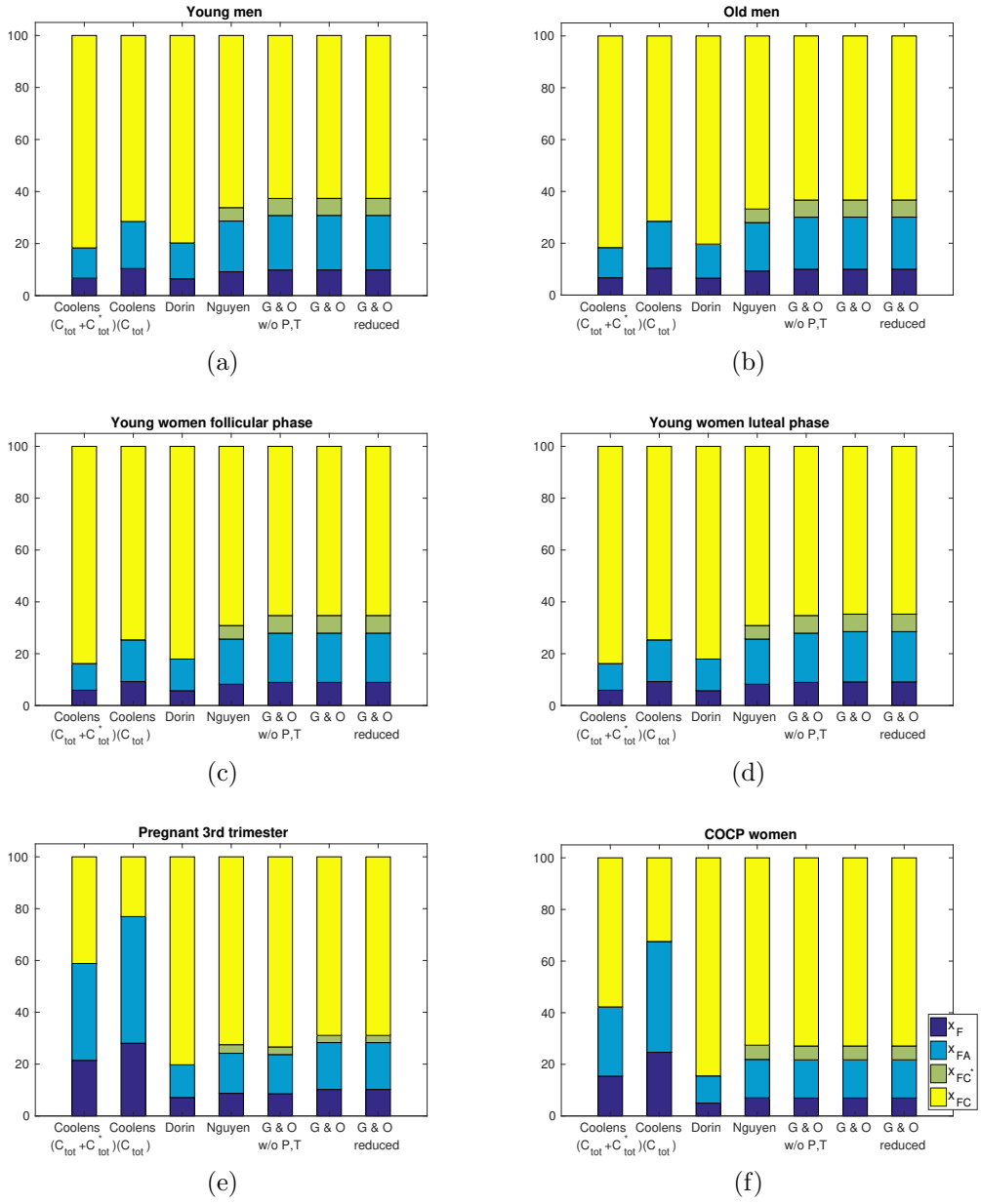



Figure 7: The model predictions of the models here and in [1], [2], and [3] differ between population groups. Estimates of $x_F$ (dark blue), $x_{FA}$ (light blue), $x_{FC^*}$ (green), and $x_{FC}$ (yellow) by Coolens et al. [1] on total CBG (Coolens $(C_{tot} + C^*_{tot})$), Coolens et al. [1] on uncleaved CBG (Coolens $(C_{tot})$), Dorin et al. [2] (Dorin), Nguyen et al. [3] (Nguyen), the static model without progesterone and testosterone (G & O w/o P,T), the static model (G & O), and the reduced static model (G & O reduced) for a) young men, b) old men, c) young women in the follicular phase, d) young women in the luteal phase, e) 3rd trimester pregnant women, and f) women taking COCP (see table 2 for input variable values). The reduced static and the static model show close to identical results.

their follicular phase (7c), young women in their luteal phase (7d), pregnant women in 3rd trimester (7e), and women taking COCP (7f) (see table 2 for input variable values).

In general the differences between the models predictions for one of our six examples varies more than the individual models predictions for each of the six examples (see 7a to 7f).

The influence of including progesterone and testosterone on the prediction of cortisol is small for both young and old men with cortisol on any form for both groups changing $< 0.03$ percentage points when comparing the results of our model with and without progesterone and testosterone (G & O and G & O reduced versus G & O w/o P,T). When comparing the predictions for young vs old men the difference in cortisol in the free form is approximately 0.14 percentage points for both the models by Dorin et al. [2], Nguyen et al. [3] and ours, while no difference is seen for the model by Coolens et al. [1]. This illustrate the effect of differences in albumin concentrations between young and old.

Even with the higher levels of total CBG and lower percentage of CBG being on cleaved form during pregnancy [60] the high level of progesterone clearly influences the distribution of cortisol on free and bound forms as seen when comparing the predictions of our static model with and without progesterone and testosterone (G & O and G & O reduced versus G & O w/o P,T) in figure 7e. When progesterone and testosterone is included in the model, the percentage of cortisol in the free form ($x_F$) is increased from 8.56% to 10.23% and bound to albumin ($x_{FA}$) from 15.14% to 18.09% for our average pregnant woman. However, for women in their luteal phase an impact of progesterone can be seen too with an increase of 0.19 percentage points of cortisol being in the free form and 0.40 percentage points bound to albumin in the predictions of our model when compared to our model without progesterone and testosterone (see G & O and G & O reduced versus G & O w/o P,T in figure 7d).

Women in 3rd trimester of pregnancy and women taking COCP (see figure 7e and 7f, respectively) are both examples of circumstances where the women have high levels of CBG and total cortisol [60]. The difference between the predictions by the model by Coolens et al. [1] and the other models are greater for these two examples than for the remaining four.

## 4. Discussion

In the present study we develop and validate a new static model for finding the concentration of free cortisol as well as determine the distribution of cortisol bound to albumin, intact and elastase cleaved CBG (CBG and CBG$^*$, respectively). We suggest directly including elastase activity $n_{CE} = \frac{X_E}{K_{CE}}$ in the calculation of free cortisol with the approximated equilibrium dissociation constant $K_{CE} = \frac{k_{C^*e}}{k^+_{C^*}} K_{M_{CE}}$ given by the Michaelis-Menten constant ($K_{M_{CE}}$), the catalytic constant ($k^+_{C^*}$), and the elimination constant ($k_{C^*e}$) for the CBG$^*$ synthesis and elimination.

If the level of elastase is unknown, but the level of CBG and CBG$^*$ is know, one is able to use the approximation $n_{CE} \approx \frac{k_{nce} C^*_{tot}}{C_{tot}}$. The results from fitting $k_{nce}$ (or more generally $n_{nce}$) individually to the data of four normal individuals in section 3.3 show that the model is able to fit data very well. The good performance of the model after fitting a common $k_{nce} = 0.63$ for all four subjects shows that the model can be used as an improved method for estimating free cortisol.

Additionally, by including the level of progesterone and testosterone in the model we are able to investigate the impact of these competitive steroids on cortisol distribution in the blood. A reduced version of the model is in the form of a fourth order polynomial and gives almost identical results as the static model for al investigations made (see section 3).

Even-though, there is a gender difference in the concentration of testosterone with normal young men having $17.7 \pm 1.0\ nM$ [49] and normal premenopausal women having $1.4 \pm 0.2\ nM$ [14] as well as an age-related change with elderly men having $12.1 \pm 0.7\ nM$ [49], our model suggests that testosterone does not influence the free cortisol concentration significantly when varied in a physiologically relevant range. On top of this, testosterone in the blood binds to sex hormone binding globulin (SHBG) [27], which is not included in the model presented here, possibly further cancelling out the potential effect testosterone could have on the free cortisol concentration.

The affinity of human CBG for progesterone is of a considerable strength [81] with dissociation constants reported in the range of 11.1 nM to 85 nM [43, 7, 23, 81]. According to Cameron et al. [23] the binding of progesterone to both albumin and CBG do not influence the level of free cortisol significantly under physiological conditions despite the apparent contest between cortisol and progesterone in the binding to the transport proteins. Cameron et al. [23] attributes this to the relatively low concentration of progesterone

relatively weak binding to both transport proteins [23]. Meyer et al. [81] argued that progesterone at high concentrations could become of importance in replacing cortisol [81]. Progesterone rises dramatically during pregnancy [12]. Meanwhile, both CBG and total cortisol rise as well with the rise in total cortisol probably being due to the rise in CBG [82]. Additionally, Nenke et al. [60] finds higher percentage of the uncleaved, high-affinity CBG in pregnant women and speculate that this could counteract the binding of progesterone [60]. Investigating these questions related to progesterone's impact, our simulations show that the static model predicts a rise in free cortisol not only when progesterone is varied independently as in section 3.2.5, but also when the parameters $X_{C0}$, $X_{A0}$, $X_{F0}$, and $n_{CC^*}$ are set to typical values seen in pregnancy (see section 3.4). Moreover, changes in predicted free cortisol and redistribution of bound cortisol are seen already at levels corresponding to levels seen for women in the luteal phase of the menstrual cycle (see section 3.2.5 and 3.4). The changes in relation to including progesterone in the model is of the same magnitude as changes due to variation of albumin in the normal range (see section 3.2.2, 3.2.5, and 3.4).

Just as Dorin et al. [2] and Nguyen et al. [3] write in regard to their models, our static model can be used to estimate the affinities of cortisol to albumin and CBG. Dorin et al. [2] estimates the $K_{FA}$, but not $K_{FC}$, since they find that their model is not particular sensitive towards changes in $K_{FC}$. Our sensitivity analysis of the model in Dorin et al. [2], Nguyen et al. [3] and the models presented here shows greater sensitivity towards $K_{FC}$ than towards $K_{FA}$ when measured in absolute and relative sensitivity as well as a sensitivity measure normalized by the normal variation seen in the literature (see 3.1). Our sensitivity analysis and further investigations of varying the different input variables and parameters show that all the models are very dependent on the default parameter values when performing local sensitivity analysis and as a consequence local parameter estimations.

Both total and intact CBG has been shown to decrease with severity of sepsis, while CBG$^*$ increased both measured in net concentration and percentage of total CBG [76]. The percentage of leucocytes circulating being neutrophils are in sepsis correlated with the relative and absolute levels of CBG$^*$ [76]. The model presented here is an equilibrium model describing the distribution of cortisol in plasma dependent on the activity of neutrophil elastase as measured in plasma. Korkmaz et al. [83] suggest neutrophil elastase as a therapeutic target [83]. By including the activity of neutrophil elastase directly in the model, we provide a method to investigate the influ-

ence of such potential therapeutics on free cortisol concentrations in plasma. However, the lowering of the affinity of CBG for cortisol by cleavage by neutrophil elastase is thought to be a mechanism of local cortisol release in tissue with inflammation [84]. The concentration of neutrophil elastase release at localised inflammatory sites may far exceed the concentration measured in circulation. Hence, local relation between intact CBG and CBG$^*$ in the interstitial compartments at sites of inflammation may differ greatly from the one measured in the circulation and, as a consequence, the level of free cortisol. The static model does not describe the concentration in the interstitial compartments, but describes the overall picture as seen in plasma. If a distinction between the levels seen in a high inflammatory interstitial compartment and the plasma compartment should be investigated, a dynamical model is needed.

In Nguyen et al. [3] a dynamic and spatially distributed model distinguishing between concentrations of cortisol and its transport proteins in the plasma and interstitial compartments is stated. Elastase is modelled only to cleave CBG in the interstitial compartment at locations with inflammation, while the synthesis of CBG$^*$ and elimination of CBG in plasma is described independent of each other and by functions not directly related to the elastase activity, but dependent on the spatial location. Free cortisol is dramatically increased locally at inflammation sites due to the elastase activity in good agreement with the underlying biological understanding. However, when relating their dynamic model to their equilibrium formula Nguyen et al. [3] leave out the terms describing CBG-cleavage and metabolite concentration gradients.

To further validate the models presented in this article combined measurements of elastase activity, CBG and total and free cortisol are needed. As described in the section 2.2.3 the activity of neutrophil elastase can be measured by an activity based assay [66], immunologically using an antibody based assay directed towards neutrophil elastase [64, 70] or immunologically using an antibody based assay directed towards the complex NE-$\alpha_1$AT [67, 68]. However, these measurements may or may not be concordant. For example Kunder et al. [66] finds higher levels of neutrophil elastase activity in combination with lower levels of $\alpha_1$-AT and an absence of an increase in the levels of NE-$\alpha_1$AT in mild and severe preeclampsia [66]. Hence, measurements of NE-$\alpha_1$AT alone would not have been a good biomarker of elastase activity in this case.

Furthermore, elastase might not be the only regulator of CBG on cleaved

and uncleaved form. In a study by Nenke et al. [85] $\alpha_1$-AT deficient subjects, who lack this native neutrophil elastase inhibitor, paradoxically have higher levels of uncleaved CBG and lower levels of CBG$^*$ [85].

Gender differences are present in the levels of the steroid hormones progesterone [8] and testosterone [9], but also in the levels of CBG [45]. The rise in cortisol and testosterone during pregnancy is often ascribed to the rise in the transport proteins CBG and sex hormone binding globulin (SHBG) [13]. However, the total concentration of cortisol is not solely controlled by the level of its transport proteins, since higher levels of CBG is seen in women compared to men [45], while higher levels of total cortisol is reported the same [86] or even higher [87] in men compared to women.

In subjects homozygote for a non-functioning CBG variant Perogamvros et al. [88] found similar free cortisol concentrations, but decreased glucocorticoid bioactivities when comparing these to subjects heterozygote for the CBG mutation and healthy controls [88]. When comparing the predictions of the different models for six example persons with estimated total levels of the input variables corresponding to different age and gender, it becomes clear that the models disagree on the distribution of cortisol in bound forms as well as their predictions for free cortisol (see figure 7). The most dramatic differences are seen in the predictions by Coolens et al. [1] for pregnant women and COCP women compared to the other models. Ho et al. [89] finds that the formulae by Coolens et al. [1] is not valid for calculating free cortisol in pregnant women [89]. Traditionally only free cortisol is considered bioactive and that the dissociations of cortisol from both albumin and CBG happen quite fast [90]. There has been speculation on a CBG receptor taking part in the activity of cortisol [91]. Hence, maybe not only the models predictions on free cortisol should be taken into consideration, but also the predictions on the distribution of bound cortisol.

Cortisol is secreted in ultradian pulses of approximately 1-2 hour period and with large amplitudes compared to their average values [92, 93]. By using a static equilibrium solution we assume that the equilibrium between the transport proteins and steroids occurs rapidly. This assumption is often applied in both methods for estimating free cortisol [2] and in dynamical models of the hypothalamic-pituitary-adrenal (hpa) axis [94]. Furthermore, by equation 1 we assume equilibrium between the elimination and enzymatic synthesis of CBG$^*$. As discussed above the activity of elastase could differ greatly between the blood and sites of inflammation. The dynamical model by Nguyen et al. [3] is in the time scale of seconds, but they do not relate their

work to the ultradian and circadian oscillations present in the system. In an ongoing work we are exploring a dynamical version of the underlying mass action model to see whether these assumptions hold and how the interaction of cortisol with plasma proteins in the blood influences the oscillating nature of cortisol.

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**Conflict of interest disclosure**

The authors report no potential conflicts of interest.

## Appendix A. Polynomial coefficients

The polynomial coefficients in equation (9a) are,

$$a_0 = p_0 p_{FC} p_{TC} p_{PC} \tag{A.1a}$$

$$\begin{aligned} a_1 =& p_0 p_{FC} p_{TC} + p_0 p_{TC} p_{PC} - p_{FC} p_{TC} p_{PC} + p_0 p_{FC} p_{PC} \\ &+ p_{CF} p_{TC} p_{PC} + p_{CP} p_{FC} p_{TC} + p_0 p_{FA} p_{TC} p_{PC} \\ &+ p_{CT} p_{FC} p_{PC} + p_0 p_{FC} p_{TC} p_{PA} + p_0 p_{FC} p_{TA} p_{PC} \end{aligned} \tag{A.1b}$$

$$\begin{aligned} a_2 =& p_{CP} p_{FC} p_{TA} + p_{CP} p_{FA} p_{TC} + p_0 p_{FC} p_{TA} + p_0 p_{FA} p_{TC} \\ &- p_{TC} p_{PC} + p_0 p_{PC} + p_{CT} p_{PC} + p_{CF} p_{PC} - p_{FC} p_{PC} \\ &+ p_0 p_{TC} + p_0 p_{FC} + p_{CF} p_{TC} + p_{CT} p_{FC} - p_{FC} p_{TC} \\ &+ p_{CP} p_{TC} + p_{CP} p_{FC} + p_{CF} p_{TC} p_{PA} + p_0 p_{TA} p_{PC} \\ &- p_{FC} p_{TA} p_{PC} + p_0 p_{TC} p_{PA} + p_0 p_{FC} p_{PA} + p_0 p_{FA} p_{PC} \\ &+ p_{CT} p_{FC} p_{PA} + p_{CT} p_{FA} p_{PC} - p_{FC} p_{TC} p_{PA} - p_{FA} p_{TC} p_{PC} \\ &+ p_{CF} p_{TA} p_{PC} + p_0 p_{FA} p_{TA} p_{PC} + p_0 p_{FC} p_{TA} p_{PA} \\ &+ p_0 p_{FA} p_{TC} p_{PA} \end{aligned} \tag{A.1c}$$

$$\begin{aligned} a_3 =& p_{CP} p_{FA} p_{TA} + p_0 p_{FA} p_{TA} - p_{FC} + p_{CP} + p_{CT} + p_{CF} \\ &+ p_0 - p_{PC} - p_{TC} - p_{TC} p_{PA} - p_{TA} p_{PC} + p_0 p_{PA} + p_{CT} p_{PA} \\ &+ p_{CF} p_{PA} - p_{FA} p_{PC} - p_{FC} p_{PA} + p_0 p_{TA} + p_0 p_{FA} \\ &+ p_{CF} p_{TA} + p_{CT} p_{FA} - p_{FC} p_{TA} - p_{FA} p_{TC} + p_{CP} p_{TA} \\ &+ p_{CP} p_{FA} - p_{FC} p_{TA} p_{PA} - p_{FA} p_{TA} p_{PC} - p_{FA} p_{TC} p_{PA} \\ &+ p_{CT} p_{FA} p_{PA} + p_0 p_{TA} p_{PA} + p_{CF} p_{TA} p_{PA} + p_0 p_{FA} p_{PA} \\ &+ p_0 p_{FA} p_{TA} p_{PA} \end{aligned} \tag{A.1d}$$

$$\begin{aligned} a_4 =& -1 - p_{FA} p_{TA} - p_{TA} p_{PA} - p_{FA} p_{PA} \\ &- p_{PA} - p_{TA} - p_{FA} - p_{FA} p_{TA} p_{PA} \end{aligned} \tag{A.1e}$$

## Appendix B. Solution formulae for the reduced static model

Several methods exist to, by help of radicals, finding analytical solutions to fourth order polynomials (also referred to as "quartics") [95]. We use the basic algorithm stated in Shmakov [95] and find the polynomial for the parameter values investigated to have four real roots of which one is positive and three are negative. Hence, the following positive root is the only physiological relevant solution to the model:

$$x_C = \frac{-g + \sqrt{g^2 - 4 \cdot h}}{2} \tag{B.1}$$

where

$$g = \frac{\frac{a_1}{a_0} - \sqrt{\left(\frac{a_1}{a_0}\right)^2 - 4 \cdot (\frac{a_2}{a_0} - y_s)}}{2} \tag{B.2a}$$

$$h = \frac{y_s - \sqrt{{y_s}^2 - 4 \cdot \frac{a_4}{a_0}}}{2} \tag{B.2b}$$

$y_s$ is a real root for the cubic equation $y^3 + b \cdot y^2 + c \cdot y + d = 0$, with

$$b = -\frac{a_2}{a_0}, \tag{B.3a}$$

$$c = \frac{a_1}{a_0} \cdot \frac{a_3}{a_0} - 4 \cdot \frac{a_4}{a_0}, \tag{B.3b}$$

$$d = -\left(\frac{a_1}{a_0}\right)^2 \cdot \frac{a_4}{a_0} - \left(\frac{a_3}{a_0}\right)^2 + 4 \cdot \frac{a_2}{a_0} \cdot \frac{a_4}{a_0}. \tag{B.3c}$$

$y_s$ can be found following the Tschirnhaus-Vieta approach [96]:

$$y_s = A \cdot cos\left(\frac{1}{3} \cdot \phi\right) + B \tag{B.4}$$

where

$$A = 2 \cdot \sqrt{\frac{-p}{3}}, \tag{B.5a}$$

$$\phi = cos^{-1}\left(\frac{3 \cdot q}{A \cdot p}\right), \tag{B.5b}$$

$$B = \frac{-b}{3}, \tag{B.5c}$$

$$p = \frac{3 \cdot c - b^2}{3}, \text{and} \tag{B.5d}$$

$$q = \frac{2 \cdot b^3 - 9 \cdot b \cdot c + 27 \cdot d}{27} \tag{B.5e}$$

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