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Comparative estrogenicity of endogenous, environmental and dietary estrogens in pregnant women II: Total estrogenicity calculations accounting for competitive protein and receptor binding and potency

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ABSTRACT

Evaluating the biological significance of human-relevant exposures to environmental estrogens involves assessing the individual and total estrogenicity of endogenous and exogenous estrogens found in serum, for example from biomonitoring studies. We developed a method for this assessment by integrating approaches for (i) measuring total hormone concentrations by mass spectrometry (Fleck et al., 2018), (ii) calculating hormone bioavailable concentrations in serum and, (iii) solving multiple equilibria between estrogenic ligands and receptors, and (iv) quantitatively describing key elements of estrogen potency. The approach was applied to endogenous (E1, E2, E3, E4), environmental (BPA), and dietary Genistein (GEN), Daidzein (DDZ) estrogens measured in the serum of thirty pregnant women. Fractional receptor occupancy (FRO) based estrogenicity was dominated by E1, E2 and E3 (ER-α, 94.4–99.2% (median: 97.3%), ER-β, 82.7–97.7% (median: 92.8%), as was the total response (TR), which included ligand specific differences in recruitment of co-activator proteins (RCA). The median FRO for BPA was at least five orders of magnitude lower than E1, E2 and E3, and three orders of magnitude lower than the fetal derived E4 and GEN and DDZ. BPA contributed less than 1/1000th of the normal daily variability in total serum estrogenicity in this cohort of pregnant women.

1. Introduction

The potential for cumulative effects of combined exposure to multiple exogenous estrogens, e.g., bisphenols, isoflavones, raloxifene, and some PCBs, particularly during early developmental exposures, has been a major focus of governmental and academic research programs (Conley et al., 2016; Dvorakova et al., 2016; Judson et al., 2015; Kunz et al., 2017; No; OECD) (Safe, 1997; Soto et al., 1997; Vilahur et al., 2014). Total estrogenicity, here defined as the total estrogenic response from all estrogens present in a sample, is a concept embraced decades ago for the evaluation of the hazards posed by xenoestrogens in environmental samples, e.g. soil and water (Avbersek et al., 2013; David et al., 2012; Fetter et al., 2014; Jarosova et al., 2014a, 2014b; Liu et al., 2015; Nie et al., 2015). The total estrogenicity concept was extended to human hazard assessment in the 1990s. Early assessments of serum estrogenicity used selective extraction and liquid chromatography to isolate fractions containing exogenous estrogens from those containing endogenous estrogens prior to screening these fractions for total estrogenicity using the E-SCREEN bioassay (Rasmussen et al., 2003; Soto et al., 1997). Overcoming a key limitation of this early work, Brouwers and co-workers directly assayed the total estrogenicity of serum using the CALUX estrogen bioassay (Brouwers et al., 2011), ensuring that total estrogenicity reflected serum concentrations, not higher or lower concentrations present in reconstituted serum extracts in prior work (Rasmussen et al., 2003; Soto et al., 1997). However, aggregation of endogenous and exogenous estrogens in the analysis and the absence of measured concentrations of chemicals in the total estrogenicity assays conducted by Brouwers et al. (2011) prevented assignment of relative

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contributions of individual or classes of estrogens to total estrogenicity. The inability to discriminate between plausible hypotheses regarding whether the chemical components increase estrogenicity in samples, e.g. increased exposure to endogenous estrogens or normal fluctuations in endogenous estrogens, was one consequence of assaying total estrogenicity without corresponding measures of serum estrogen concentrations. To date, these and other challenges have been obstacles to development of accepted methods for in situ measurement of estrogenic activity in intact organisms, and a need for alternative approaches.

The use of in vitro systems for measurement of total estrogenicity remains attractive because it directly captures multiple dimensions of estrogenicity - receptor affinity, potency (co-activator recruitment (Jeyakumar et al., 2011)) and subsequent events leading to gene transcription or non-genomic signaling, and the potential for synergism and antagonism. Yet other outcomes important for public health protection, e.g. measuring the relative contribution of specific chemicals to total estrogenicity in the context of multiple estrogens, and rapid assessment of relative estrogenic potential and total estrogenicity from human chemical biomonitoring data, are not addressed with these approaches. For example, as human biomonitoring expands through programs like NHANES (National Health and Nutrition Examination Survey) and CHEAR (Children's Health Exposure Analysis Resource), there will be a growing need for screening level approaches to assess the estrogenic potential for an increasing range of chemicals found in blood, and to understand how the presence of high concentrations of endogenous estrogens influences the estrogenicity of these compounds. In addition, added estrogens may contribute greater estrogenicity in test systems with lower background estrogens (e.g. in vitro screening models) compared with systems with higher background estrogens (Hwang et al., 2006), for example during pregnancy. The introduced estrogen must both compete with multiple estrogens for access to tissue receptors and overcome already higher levels of receptor occupancy to contribute a meaningful increment in estrogenicity (law of mass action and (OECD)) or antagonize the action of more potent estrogens present (Gerard et al., 2015; Jarosova et al., 2014a; Rajapakse et al., 2004). To our knowledge, there has been limited consideration of how the high estrogen status of normal human pregnancy could influence the relative estrogenicity of environmental estrogens, in particular, whether the high estrogen background impacts the exposure conditions required to cause adverse effects. Thus, there is a need for new approaches that allow estimation of total and relative estrogenicity in human biofluids in contact with tissues from measured concentrations derived from biomonitoring studies. Such an approach should consider multiple mechanistic aspects of estrogenicity and concepts of hormone bioavailability that are commonly used in clinical endocrinology and can be founded on measured serum concentrations of estrogens.

To better understand the endocrine disrupting potential of chemicals in humans, we frame the question of the estrogenicity of exogenous estrogens from a clinical perspective, in the biological context of an intact open system of multiple circulating estrogens. We hypothesize that synthetic and/or dietary estrogens must contribute sufficient “added” estrogenicity to shift total estrogenicity during pregnancy by a significant increment of normal intra-individual daily variability to be considered abnormal. We explore the magnitude of “added” estrogenicity through application of a new approach for screening the estrogenicity of compounds that embraces extrinsic and intrinsic factors influencing potency (Fig. 1) and thus total and relative estrogenicity. The overall approach represents a framework for calculating estrogenicity in serum extendable to any ligand-receptor system.

2. Materials and methods

All human subject research activities were conducted in accordance with protocols approved by the Pacific Northwest National Laboratory Institutional Review Board (IRB # 2014–14). The participation of the National Center for Toxicological Research was reviewed and approved by the Food and Drug Administration (FDA) Research Involving Human Subjects Committee (RIHSC#10–119T).

2.1. Overview of method

In the proposed approach, starting with the measured concentrations of the ligands in serum, the protein-binding of ligands is simulated to yield the serum bioavailable concentrations of ligands (unbound + albumin bound) (Fleck et al., 2018), which is then used to calculate receptor occupancy for each ligand by numerically solving for the multiple equilibria between the bioavailable ligands and cellular estrogen receptors. The relative response for each ligand is subsequently calculated based on receptor occupancy and relative co-activator affinity (RCA) - a measure of relative potency that represents intrinsic factors influence potency down-stream of receptor binding (Jeyakumar et al., 2011). Applying this approach to the measured concentrations of seven estrogens, which include estrone (E1), estradiol (E2), estriol (E3), estrone (E4), genistein (GEN), diadzein (DDZ), and bisphenol A (BPA), measured in a 30 member cohort of pregnant women (Fleck et al., 2016, 2018; Teeguarden et al., 2016), we report the relative estrogenicity of each, compare the contribution of each to total estrogenicity, and compare the contribution to the variability in estrogenicity in non-pregnant women.

2.2. Experimental design and general framework for simulations

We have adapted long-standing gold-standard approaches for measuring total hormone concentrations by mass spectrometry (Fleck et al., 2018), mathematically rigorous methods for solving complex equilibria between multiple ligands, serum proteins, and estrogen receptors, and more recent approaches for quantitatively describing determinants of estrogen potency, for calculation of relative and total estrogenicity in serum or cells where multiple estrogenic compounds are present.

The estrogenic potency of ER-α and ER-β ligands depends on various extrinsic factors such as absorption, distribution, metabolism and elimination (ADME) and intrinsic properties related to individual elements of the mechanism of action, like receptor binding. The extrinsic factors influence potency by affecting the extent and magnitude of exposure. Compounds with properties that produce higher concentrations for a given exposure—low metabolism, high absorption—have higher potency than compounds with lower absorption rates and higher metabolism. Similarly, binding of compounds to serum proteins that limit the bioactive concentrations to the unbound or unbound and weakly bound fractions is a key extrinsic factor influencing exposure at the target site (tissue, cell cytosol). For example, steroid hormones and molecules with similar structures are bound with varying degrees of affinity to serum proteins, most notably albumin and sex hormone binding globulin (SHBG). When binding affinity is sufficiently high, the bound fraction of the molecule has limited access to cells and cellular receptors (Egleston et al., 2010; Pardridge et al., 1980; Pardridge, 1981). For the steroid hormones testosterone and estradiol, experimental evidence supports the hypothesis that the bioavailable fraction capable of entering cells consists of the unbound and albumin bound molecules (Belgorosky et al., 1987; Egleston et al., 2010; Pardridge et al., 1980; Pardridge, 1981). Molecules bound to SHBG with high affinity, have limited bioavailability under most conditions (Egleston et al., 2010; Pardridge et al., 1980; Pardridge, 1981). For clinical research, diagnosis of endocrine disorders, and exposure assessment, there is a recognized preference for use of “free” or “biologically active” over total concentrations of steroid hormones (Belgorosky et al., 1987; Hackbarth et al., 2011; Sartorius et al., 2009) and related molecules (Armitage et al., 2014; Gülden and Seibert, 2003; NRC, 2017; Teeguarden and Barton, 2004; Teeguarden et al., 2005). In this case, free refers to non-protein bound and bioavailable refers to the sum of free compound and compound bound to albumin.

For effects mediated by mechanisms involving ligand binding to

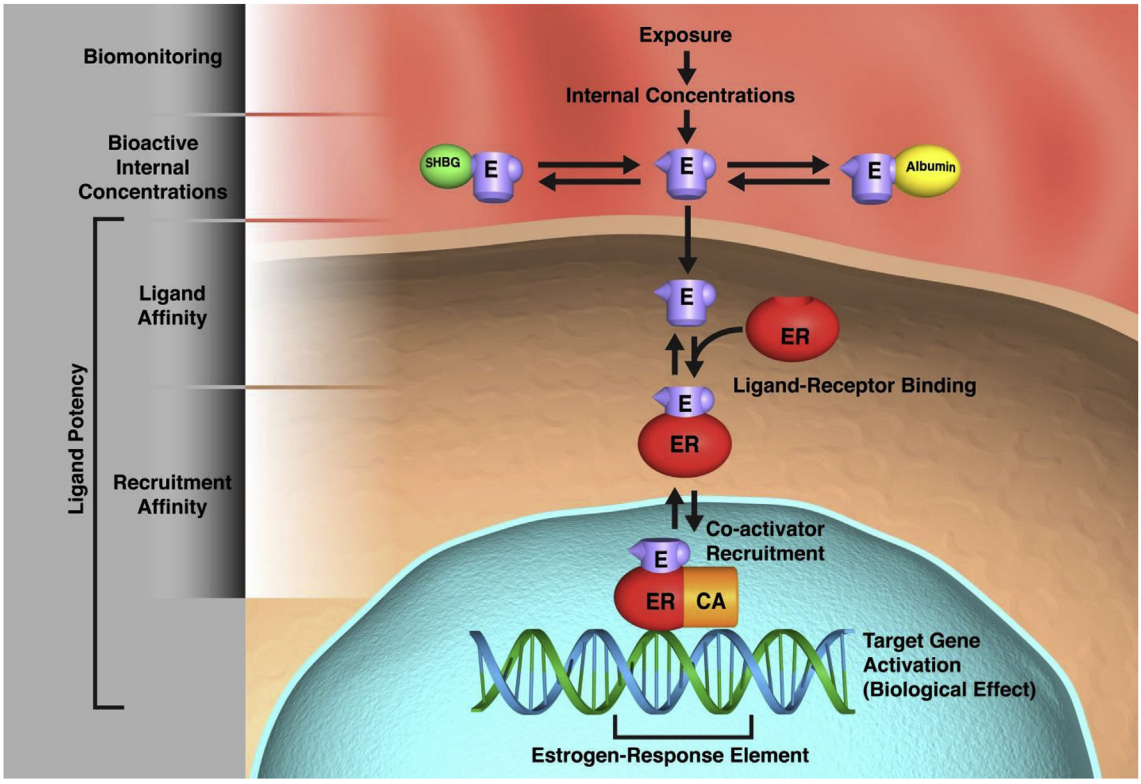

Fig. 1. Diagram of the exposure-to-relative estrogenicity calculations. Bioavailable concentrations estrogens (E) are calculated by solving for the complex equilibrium with serum proteins (Albumin), Steroid Hormone Binding Globulin (SHBG). Bioavailable estrogens then compete for estrogen receptor (ER). The effectiveness of coactivator protein (CA) recruitment is then considered as a key element of relative potency of each estrogen.

receptors, ER-α and ER-β, in our case, receptor binding is the first step in the sequence events which together comprise the intrinsic factors influencing ligand potency. Subsequent to the formation of the estrogen-receptor complex, proteins are recruited to the complex, forming an estrogen-receptor-coactivator complex (Jeyakumar et al., 2011). The co-activator complex induces estrogenic effects through transcriptional activation (Jeyakumar et al., 2011) (Fig. 1). Ligand-receptor dissociation constants, the standard measure of receptor affinity, are commonly measured using routine laboratory methods. Affinity constants, or more commonly, relative binding affinities, have been reported for many xenoestrogens (Jeyakumar et al., 2011; Jiang et al., 2013; Kuiper et al., 1997; Zhu et al., 2006). More recently, a second intrinsic factor of ligand potency, the affinity of the receptor-ligand complex for co-activator protein complexes was measured for a series of endogenous and exogenous estrogens by Katzenellenbogen and co-workers (Jeyakumar et al., 2011).

The proposed modeling framework, uses accepted experimental mathematical approaches adaptable to any number of compounds and measures of ligand and receptor characteristics, some of which, such as, receptor affinity and receptor concentrations are easily obtained, while others, such as RCA are more difficult to obtain (Jeyakumar et al., 2011). Furthermore, other intrinsic factors such as differential affinity of the ligand-receptor-co-activator complex for genomic transcription factor binding sites, may also contribute to the overall estrogenic potency of ligands. These factors are however not considered in our model because they are difficult to measure and quantify.

2.3. Volunteer selection and demographics

Thirty pregnant volunteers were recruited for the study from the Salt Lake City, UT metropolitan area in 2014. Details of the recruiting and volunteer selection process were reported previously (Teeguarden et al., 2016). All enrolled volunteers completed the study. The average age of the thirty volunteers was 26.5 years (range 18–34). The average weight of volunteers was 71.0 kg (range 49.8–98.0 kg). The average stage of pregnancy was 23.7 weeks (range 15–34). Demographic information for each volunteer, BPA exposure levels and isoflavone exposure levels are reported in previous publications (Fleck et al., 2016; Teeguarden et al., 2016).

2.4. Exposure scenarios considered

The comparative estrogenicity of exogenous and endogenous estrogens was evaluated in four scenarios of increasing completeness (Table 1). These scenarios allow dissection of the contributions that the endogenous and exogenous estrogens make to total estrogenicity.

Table 1
Exposure scenarios for total serum estrogenicity evaluation.

Scenario	Description	Ligands[a]
1	Primary Endogenous Estrogens	E1, E2. E3
2	Primary Endogenous Estrogens and BPA	E1, E2, E3, BPA
3	Primary Endogenous and Dietary Estrogens	E1, E2, E3, BPA, GEN, DDZ
4	Primary, Dietary and Fetal Derived Estrogens	E1, E2, E3, BPA, GEN, DDZ, E4

[a] Serum estrogen concentrations were obtained from Fleck et al. (2018).

Scenario 1 evaluates the relative and total estrogenicity of only endogenous estrogens E1, E2, and E3, for which reliable serum concentration measurements could be obtained the study cohort. The relative contribution of BPA to serum estrogenicity, in the presence of endogenous estrogens is evaluated in Scenario 2. Scenario 2 utilizes measured or reliably estimated unconjugated BPA serum concentrations from the cohort. Serum BPA concentrations were estimated by using measured urine values and empirical relationships derived from human studies as described previously (Teeguarden et al., 2016). Scenario 3 expands on Scenario 2 by considering two phytoestrogens, namely GEN and DDZ, in addition to E1, E2, E3, and BPA. Serum aglycone GEN and DDZ concentrations were estimated from total concentrations using the aglycone:total ratios measured in the volunteer cohort. Finally, Scenario 4 represents the most complete case, where all seven estrogens (E1, E2, E3, E4, GEN, DDZ, and BPA) are considered. When serum E4 concentrations were below limits of detection (all but one volunteer), serum concentrations were estimated from measured total E4 (E4 plus conjugates), as described in a companion paper (Fleck et al., 2018).

Total serum estrogenicity was also calculated for three phases of the menstrual cycle in non-pregnant women, based on literature reporting of serum concentrations values (Chatzidimitriou et al., 2015; Reed and Carr, 2015). These calculations were included to provide additional biological context for serum estrogenicity calculated during pregnancy.

2.5. Unbound and bioavailable estrogen concentrations

For the steroid hormones testosterone and estradiol, experimental evidence supports the hypothesis that the bioavailable fraction capable of entering cells consists of the unbound and albumin bound molecules (Belgorosky et al., 1987). Molecules bound to SHBG are not bioavailable under most conditions. For clinical research, diagnosis of endocrine disorders, and exposure assessment, there is a recognized preference for use of “free” or “biologically active” over total concentrations of steroid hormones (Belgorosky et al., 1987; Hackbarth et al., 2011; Sartorius et al., 2009) and related molecules (Armitage et al., 2014; Gülden and Seibert, 2003; NRC, 2017; Teeguarden and Barton, 2004; Teeguarden et al., 2005).

While mass spectrometry is the preferred method for measuring total serum concentrations of steroid hormones (Handelsman and Wartofsky, 2013), bioavailable concentrations of hormones are measured experimentally by ultracentrifugation dialysis (Hackbarth et al., 2011; Vermeulen et al., 1999) or ammonium sulfate precipitation methods (Vermeulen et al., 1999). Both methods are reliable but costly and labor intensive, and in the case of ultrafiltration dialysis, not without challenges, due to high assay variability (Vermeulen et al., 1999). For more than two decades, an attractive and common alternative to direct measurement is the calculation of bioavailable fractions using measured total concentrations of hormones, their affinity constants for serum binding proteins and the corresponding concentrations of the binding proteins (Belgorosky et al., 1987; Dunn et al., 1981; Egleston et al., 2010; Sartorius et al., 2009; Vermeulen et al., 1999).

In the present study, the bioavailable (free and albumin-bound) concentrations of the estrogens of interest were calculated by using measured total concentrations of hormones, their affinity constants for serum binding proteins and the corresponding concentrations of the binding proteins (Belgorosky et al., 1987; Dunn et al., 1981; Egleston et al., 2010; Sartorius et al., 2009; Vermeulen et al., 1999), according to the principles of mass action, formulated into a mass-balance model that treats the competitive binding between different ligands and proteins, as described in the next section.

2.6. Metrics of estrogenicity

Potency is a common term used for describing the activity of drugs, including those operating through receptor-mediated mechanisms. For estrogen receptor based mechanisms, Jeyakumar and co-workers describe potency as comprising two factors: (i) affinity of ligand binding to the receptor and (ii) affinity of the co-activator recruitment to the estrogen receptor-ligand complex (Jeyakumar et al., 2011). In this work, we adopt this concept to describe the potency of individual ER ligands (referred to as estrogenicity) in terms of the combined effect of receptor affinity characterized by the disassociation constant (K_d) for the ligand-receptor pair and a second term, the relative coactivator affinity (RCA) that reflects the affinity of coactivator for the estrogen receptor-ligand complex. Other factors, such as affinity for the transcription binding sites, or efficacy of transactivation are not considered in our study due to the lack of information available in the literature regarding these factors.

To assess the cumulative estrogenicity of a group of ER ligands in serum, we report total and relative estrogenicity as a function of calculated receptor occupancy alone, and a function of calculated receptor occupancy multiplied by RCA. The first metric, fractional receptor occupancy (FRO), represents the combination of the relative affinities of each estrogen and their measured concentrations in serum. We utilize available data on ER-α and ER-β receptor affinities, ligand concentrations and bioavailability to calculate total and fractional receptor occupancy as a measure of estrogenicity. The second metric, total response (TR) is the product of receptor occupancy and experimentally derived or assumed (within probable bounds) values of RCA. We refer to the second metric of estrogenicity as total response, to differentiate it from estrogenicity calculated as receptor occupancy alone (i.e. FRO).

Disassociation constants, used to calculate FRO, are routinely measured and available for all estrogens evaluated in this study. Likewise, the values of relative co-activator affinities for ER-α and ER-β for E1-E3, GEN and DDZ, but not BPA or E4, used in this study, were taken from Jeyakumar et al. (2011); Jiang et al. (2013). The RCA value for E4 was assumed to be similar to E3, due to their structural similarities. The data from Jayakumar and co-workers are consistent with bounds for the intrinsic factors influencing relative potency of estrogens being between 0.1% and no more that 200%. We elected to use these bounds to explore the impact differences in potency on the contribution of BPA to total serum estrogenicity in our cohort. For BPA, a reasonable lower and upper bounds of 20 and 500 (1/5 and 5 times RCA of E2), were assumed. The actual values of RCA for the various estrogens are tabulated in Table 2.

2.7. Mathematical framework for calculations

The mathematical model used in this study to simulate the various processes starting from the protein-binding of ligands in the plasma to the eventual ligand binding to the cellular estrogen receptors can be conceptually broken down into three main parts. The first set of rate equations (Eq. (1)) describe the binding of the ligand to serum proteins in the plasma compartment. Subsequently, the bioavailable (free + albumin-bound) fraction of the ligand is transported through the cellular membrane to be made available for binding to the estrogen receptors in a cell (Eq. (2a)). This process, in our simulation, is modeled as a passive diffusion process, with a sufficiently large diffusion constant to ensure near-instantaneous equilibrium between the bioavailable concentrations of the ligands in the plasma and cellular compartments (Eq. (2b)). Eventually, the binding of the bioavailable ligand in the cellular compartment to the estrogen receptors is described by the final set of rate equations (Eq. (3)).

$$L^{free} + Albumin^{free} \overset{K_d^{L:Alb}}{\Leftrightarrow} L{:}\,Albumin \tag{1a}$$

$$L^{free} + SHBG^{free} \overset{K_d^{L:SHBG}}{\Leftrightarrow} L{:}\,SHBG \tag{1b}$$

$$L^{bioavailable} = L^{free} + L{:}\,Albumin \tag{2a}$$

Table 2
Dissociation constants and RCA values.

Ligand	K_d (nM)				RCA	
	SHBG	Albumin	ERα	ERβ	ERα	ERβ
Estrone (E1)	6.67[a]	50000[a]	1[j]	20[j]	3[g]	28.1[g]
17β-Estradiol (E2)	2.12[b]	20700[b]	0.1[f]	0.4[f]	100[g]	100[g]
Estriol (E3)	230[a]	25000[a]	0.9[j]	1.1[j]	95.7[g]	149[g]
Estetrol (E4)	10000000[i]	10000000[i]	2.5[j]	13.3[j]	100[m]	150[m]
Genistein (GEN)	1590[c]	7800[d]	6.25[k]	0.4[k]	9.2[g]	36.2[g]
Diadzein (DDZ)	10000000[i]	26000[d]	50[k]	22.2[k]	0.88[h]	54.4[h]
Bisphenol A (BPA)	13900[c]	350000[e]	200[l]	133.3[j]	20/500[n]	20/500[n]
Testosterone (TES)	0.625[a]	40000[a]	–	–	–	–

[a] (Dunn et al., 1981).
[b] (Rosner, 2015).
[c] (Dechaud et al., 1999).
[d] (Bolli et al., 2010).
[e] (Xie et al., 2010).
[f] (Kuiper et al., 1997).
[g] (Jeyakumar et al., 2011).
[h] (Jiang et al., 2013).
[i] Non-binder, assigned a reasonably large value.
[j] Estimated from RBA obtained from Zhu et al. (2006).
[k] Estimated from RBA obtained from Jeyakumar et al. (2011).
[l] Estimated from RBA obtained from Kuiper et al. (1997).
[m] Due to structural similarity with E3, assumed values closer to that of E3.
[n] Simulations were performed for the two extreme bounds of 20 and 500 (1/5 and 5 times the RCA of E2) assumed for BPA.

$$L_{plasma}^{bioavailable} \overset{Diffusion}{\leftrightarrow} L_{cell}^{bioavailable} \tag{2b}$$

$$L^{bioavailable} + ER\alpha^{free} \overset{K_d^{L:ER\alpha}}{\Leftrightarrow} L^{bioavailable}{:}\, ER\alpha \tag{3a}$$

$$L^{bioavailable} + ER\beta^{free} \overset{K_d^{L:ER\beta}}{\Leftrightarrow} L^{bioavailable}{:}\, ER\beta \tag{3b}$$

The total ligand and plasma protein concentrations used in our simulations are tabulated in Supplemental Table 1 for each subject. The dissociation constants for ligand binding to plasma proteins, SHBG and Albumin, and the two estrogen receptors, ER-α and ER-β, denoted by $K_d^{L:SHBG}$, $K_d^{L:Alb}$, $K_d^{L:ER\alpha}$, and $K_d^{L:ER\beta}$, respectively, are provided in Table 2. In our analyses, in addition to the scenario dependent estrogens, testosterone was also considered as a ligand competing for binding sites on the two main binding plasma proteins, namely, the SHBG and albumin. Belgorosky et al. (1987) and Moll et al. (1981) showed that including testosterone is required for an accurate calculation of bioavailable concentrations of other steroid hormones which also bind to SHBG and albumin (Belgorosky et al., 1987; Moll JR et al., 1981). The estrogen concentrations were either directly measured or estimated from experimentally measured values, as described in an earlier publication (Fleck et al., 2018). Also, as noted in Supplemental Table 1, the gestation week dependent values of plasma testosterone and SHBG values were obtained from the data published in O'Leary et al. (1991), whereas, the total plasma albumin concentration was assumed to be independent of gestation week and was fixed at 4×10^5 nM. Moreover, based on a previously published study (Teeguarden and Barton, 2004), the total receptor concentration for both ER-α and ER-β was fixed at 59 nM. Finally, the volumes of the plasma and cellular compartments were assumed to be equal to the blood and cellular volumes, 5 L and 1×10^{-7} μL, respectively.

These systems of simultaneous multiple equilibria described in Eqs (1)–(3) were numerically solved in AcsIX. In the simulation, the forward and backward rate equations are described in terms of on- and off-rates as described next.

For a general ligand-receptor binding equation (which could represent either ligand-protein binding or estrogen-receptor binding):

$$L + R \overset{K_d}{\Leftrightarrow} L{:}\, R \tag{4}$$

The kinetics of forward and backward reactions can then be described by on- and off-rates:

$$L + R \overset{k_{on}}{\rightarrow} L{:}\, R \tag{5a}$$

$$L{:}\, R \overset{k_{off}}{\rightarrow} L + R \tag{5b}$$

The instantaneous rate of change of concentrations of the ligand ($[L]$), receptor ($[R]$), and the ligand-receptor complex ($[L{:}\, R]$) are related by the on/off-rates as follows:

$$\frac{d[L{:}\, R]}{dt} = -\frac{d[L]}{dt} = -\frac{d[R]}{dt} = k_{on}[L][R] - k_{off}[L{:}\, R] \tag{6}$$

At equilibrium, the rate of change of concentration of different species is zero, which means that:

$$k_{on}[L][R] - k_{off}[L{:}\, R] = 0 \Rightarrow \frac{k_{off}}{k_{on}} = \frac{[L][R]}{[L{:}\, R]} \tag{7}$$

The last term in the equation above represents the dissociation constant, k_d, which yields the following relationship between the on- and off-rates, and the dissociation constant:

$$K_d = \frac{k_{off}}{k_{on}} \tag{8}$$

The equilibrium concentrations of ligand ($[L]_{eq}$), receptor ($[R]_{eq}$), and ligand-receptor complex ($[L{:}\, R]_{eq}$), can then be obtained by integrating the above equations over a sufficiently long period of time (T):

$$[L{:}\, R]_{eq} = \int_0^T (k_{on}[L][R] - k_{off}[L{:}\, R])\, dt \tag{9a}$$

$$[L]_{eq} = [R]_{eq} = \int_0^T (k_{off}[L{:}\, R] - k_{on}[L][R])\, dt \tag{9b}$$

2.8. Fractional receptor occupancy (FRO) and total response (TR) metrics

FRO and TR metrics were calculated based on the equilibrium concentrations of the ligand-receptor complexes obtained from the simulations as described in the previous section. Consider a scenario, where there are a total of N ligands, denoted by L_1, ...,L_n, ...L_N, and let $[L_n{:}\, ER]_{eq}$ denote the equilibrium concentration of the ligand-receptor complex for the nth ligand and a general ER receptor (ER-α or ER-β in our simulations), then the FRO metric for the nth ligand and ER receptor is defined as:

$$\text{FRO for } L_n = \frac{[L_n{:}\, ER]_{eq}}{[ER]_{tot}}, \tag{10}$$

where, $[ER]_{tot}$ denotes the total receptor concentration.

Likewise, the TR metric for the nth ligand is defined as:

$$\text{TR for } L_n = \text{FRO of } L_n \times \text{ RCA of } L_n. \tag{11}$$

Additionally, we can also define relative response of a ligand by normalizing its TR by the sum of TR for all N ligands:

$$\text{RR for } L_n = \frac{\text{TR of } L_n}{\text{TR of } L_1 + \text{ TR of } L_2 + ... + \text{TR of } L_N}. \tag{12}$$

2.9. Definition of "meaningful increment" of total estrogenicity

For purposes of this study, we elected to define a "meaningful increment of total estrogenicity" biologically, not statistically, as a small

Table 3
Summary statistics for bioavailable concentrations in nM for various ligands in pregnant women (Scenario 4, ER-α).

Estrogen	Median	Min	Max	IQR
E1	1.9	0.26	16	2.4
E2	2.6	1.1	9.3	1.9
E3	7.8	1.4	33	6.6
E4	2.7E-02	6.3E-03	0.25	2.9E-02
GEN	3.7E-02	6.0E-03	2.8	0.17
DDZ	3.9E-02	2.8E-03	3.3	0.15
BPA	6.9E-05	2.0E-06	4.3E-03	1.4E-04
TES	9.1E-02	8.4E-02	0.13	1.3E-02

fraction of normal variability in serum estrogenicity reported in our study. We assume here that shifting total estrogenicity by less than 1/1000th (0.1%) of measured normal daily variability would be biologically insignificant. While our selection of 0.1% of normal variability as the definition of biologically meaningful increment in total estrogenicity must be seen as arbitrary, it is a) a very small increment of estrogenicity (conservative) calculated in our cohort, which is also supported by a well-established base of normal variability in estrogen concentrations in pregnant women (Magiakou et al., 1996b; Oakey, 1979; Reck et al., 1979); b) can be replaced by other selected values for re-interpretation of the results here, and c) is appropriate for its use here as a screening and prioritization method for human exposure to endogenous estrogens.

3. Results

3.1. Calculated bioavailable concentrations

The bioavailable concentrations and fractions ((unbound + albumin-bound)/total) for the 30-member cohort are presented in Tables 3 and 4, respectively. The median bioavailable fraction ranged from 0.02 to 1.0. BPA, DDZ, GEN, E3 and E4 had bioavailable fractions exceeding 0.9, much larger than E1 and E2 and TES. The bioavailable concentrations of the three main endogenous estrogens (E1, E2, and E3) were found to be the highest followed by the two isoflavones (GEN and DDZ) and E4, and finally BPA, which had the lowest bioavailable concentrations (Table 3, Fig. 2). Based on the median bioavailable concentration values (Table 3), the ranking for bioavailable concentrations in serum were: E3 > E2 > E1 > DDZ > GEN > E4 > BPA. This ranking was slightly different than the ranking based on the total plasma concentrations: E2 > E1 > E3 > DDZ > GEN > E4 > BPA (Complete data sets for total concentrations, unbound, albumin bound and SHBG bound and summary statistics for total concentrations are provided in Supplemental Tables 1–5). The bioavailable fraction of E2 was the lowest (median: 0.11), exceeded slightly by E1 (median: 0.15), while the other estrogens were almost fully bioavailable (unbound or bound to albumin). The bioavailable fraction of each estrogen is summarized in Table 4.

Table 4
Summary statistics for bioavailable fraction of the total concentration for each hormone ligand in pregnant women (Scenario 4, ER-α).

Estrogen	Median	Min	Max	IQR
E1	0.15	0.14	0.20	1.6E-02
E2	0.11	0.10	0.15	1.3E-02
E3	0.92	0.91	0.94	8.9E-03
E4	1.0	1.0	1.0	2.4E-07
GEN	1.0	1.0	1.0	5.6E-04
DDZ	1.0	1.0	1.0	2.5E-08
BPA	0.99	0.99	0.99	1.3E-03
TES	2.0E-02	1.8E-02	2.7E-02	2.5E-03

3.2. Estimated fractional receptor occupancy and total and relative response

During the third trimester of pregnancy, within the study cohort, the FRO-based ranking for the three endogenous estrogens E1, E2, and E3 was consistently E2 > E3 > E1, for ER-α, and E3 > E2 > E1, for ER$-\beta$, while for the dietary and synthetic estrogens, the ranking was GEN > DDZ > BPA (Table 7, Fig. 3) across all exposure scenarios. The serum total estrogenicity assessed as fractional receptor occupancy, for the most general case of Scenario 4, was dominated by E2 (median: 67% for ER-α and 42% for ER-β), E3 (median: 25% for ER-α and 48% for ER-α) and E1 (median: 5.3% for ER-α and 0.57% for ER-β). The median fraction of receptors occupied by BPA was consistently five or more orders of magnitude lower than E1, E2 and E3, and three orders of magnitude lower than the fetally derived E4 and GEN and DDZ (Fig. 3, Table 7). Repeated measures of serum estrogens over the study period revealed substantial variability in total ER-α and ER-β receptor occupancy (Fig. 3, Table 7). The observed normal variability in total FRO was the result of fluctuations in endogenous estrogens (Scenario 1), with minimal contributions from dietary or environmental estrogens.

Based on the median TR values for Scenario 4, (Fig. 4, Table 8), both E2 and E3 contributed significantly to TR though ER-α and ER-β. E2 was the most potent serum estrogen during the gestational periods evaluated for ER-α receptor (median TR value: 6.7E + 03), whereas, E3 was the most potent for ER-β receptor (median TR value: 7.1E + 03). The median TR values for E1 and E4, were consistently less than 17 for both ER-α and ER-β. One dietary estrogen, GEN, had a median TR of 21 for ER-β, somewhat higher than the value for E1. Median TR values for the dietary and synthetic estrogens DDZ and BPA were remarkably lower. The low TR values for these estrogens reflect the combination of lower measured serum concentrations, lower receptor affinities and RPFs assumed to be equivalent to measured RCA's which are also lower (except BPA and E4 where RCA's are empirically chosen or assumed). Without measured RCA's for BPA, we elected to use values that represented an assumption that BPA is similar to other lower affinity estrogens, and we also explored the other extreme, BPA being significantly more potent than E2, though there is no evidence of this when BPA acts through ER-α and ER$-\beta$. Conclusions here about the TR for BPA thus include use of RCA values of 20 (1/5th of E2), and 500 (5 times E2), reflecting these assumptions. Under these conditions, the median TR values for BPA for the two estrogen receptors were 3–8 orders of magnitude lower than those for the endogenous estrogens, and 1–2 orders of magnitude lower than GEN and DDZ. Values of the TR, normalized to total receptor occupancy (relative response) are tabulated in Supplemental Tables 6 and 7, and plotted in Supplemental Fig. 1.

Calculated TR ranged from 8.3E + 03 to 9.5E + 03 (ER-α) and from 9.3E + 03–1.3E + 04 (ER-β) in Scenario 1 (endogenous estrogens only). Daily variability in median TR across all volunteers (endogenous estrogens only, Scenario 1, IQR/Median) was 4.4% (ER-α)-8.7% (ERβ). Addition of dietary and environmental estrogens (Scenario 4) had a minimal ($\ll$1%) impact on the median TR (Table 8).

As a comparator for pregnancy, total receptor occupancy were calculated using literature values for serum ranges measured during the follicular, ovulatory and luteal phases of the menstrual cycle in the middle to upper range of the occupancy curve, a region generally associated with linear increases in occupancy and biological response (Table 5). Occupancy was highest during the ovulatory phase (89%, ER-α, 66% ER-β), and ranged from 67 to 89% (ER-α) and 35–66% (ER-β). Bioavailable concentrations of E1, E2 and E3 (Table 6) were much lower overall during all three phases than the measured values in our cohort for the third trimester (Table 3).

3.3. Biological relevance of environmental and dietary estrogens

The absolute values for biological meaningful contributions to total estrogenicity were calculated as 0.1% of the 1.8% (ER-α)-4.7% (ER-β)

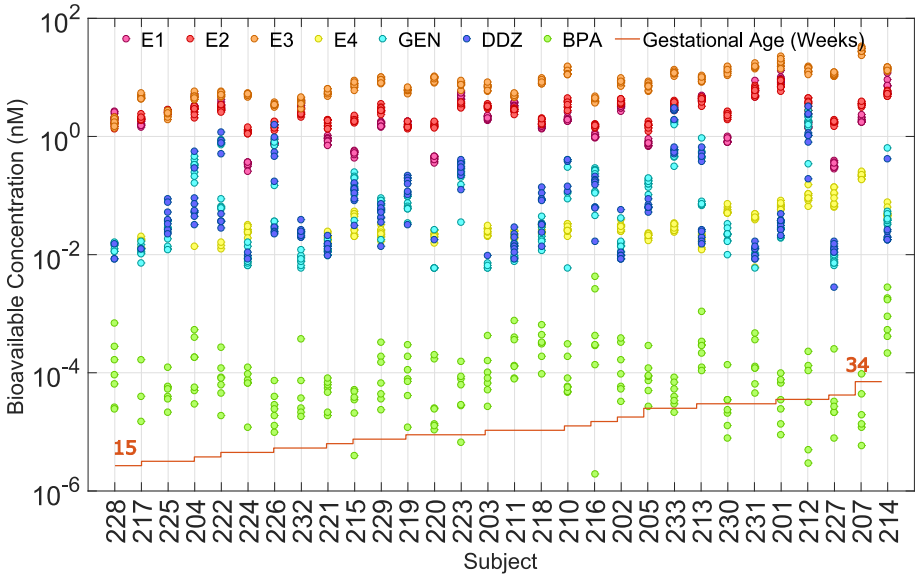

Fig. 2. Calculated bioavailable concentrations of the seven estrogens measured in the serum of the 30 member cohort. Each point represents one of up to seven measurements taken over the course of a day. Gestation age increases from left to right.

variability in FRO (as IQR/Median, Table 4), or 4.4% (ER-α)-8.7% (ER-β) variability in median TR in our cohort. Thus, increasing FRO by $\leq$ 0.0018% for ER-α or $\leq$0.0047% for ERβ, or TR by $\leq$ 0.39 for ER-α or $\leq$1.0 ERβ would indicate biologically insignificant contributions to estrogenicity by our criteria. Based on calculated FRO's, and TR's, the contribution of BPA to total estrogenicity was several orders of magnitude lower than the 1/1000th of the normal variability in our cohort criteria for biological meaningful contributions to total estrogenicity (Table 7, Table 8), even when we assumed the RCA for BPA was five times greater than E2. In contrast, measured serum concentrations of GEN and DDZ always exceeded our criteria, in the case of GEN, by two orders of magnitude for ERβ.

4. Discussion

As human biomonitoring, laboratory screening of chemicals for estrogenicity, and research into mechanisms of action continue to rapidly expand, deriving knowledge for regulation and mitigation from the intersection of these three research areas will remain a grand challenge in public health. The focus on chemical screening and biomonitoring has led to substantial improvements in the efficiency and accuracy of the supporting technologies; yet, surprisingly, there has been comparatively little effort and fewer advancements in approaches for placing human relevant exposures obtained by biomonitoring in the context of the quantitative information on the potency of estrogens measured in laboratory screenings. Without rigorous and quantitative comparisons between measured exposures and laboratory assessments of potency, subjective conclusions about risk, or the nature of the potential hazard are drawn from biomonitoring data (Teeguarden et al., 2013) without the rigorous evidentiary support common in other scientific disciplines, like engineering and pharmacology. A good example of this was recently published. An administered dose of 833 mg/kg of BPA, 833,000 times higher than daily human exposure (European Food Safety Authority, 2013; Teeguarden et al., 2013; U.S. Food and Drug Administration, 2008), delivered by subcutaneous implant, was used to induce effects in mice, which were reported as evidence that BPA is implicated in similar effects in humans (Nicholson et al., 2018). No objective comparison of external or measured internal exposures, doses, or routes of administration were made to support the author's assertion of relevance to either human exposure or disease. We address two overarching challenges related to assessing the significance of measured serum concentrations of environmental estrogens: (i) human biomonitoring of xenoestrogens that routinely excludes measurement of the most potent, ubiquitous endogenous estrogens; (ii) biologically supported, quantitative criteria and supporting metrics for "significant estrogenicity" required to disrupt biological systems.

Biomonitoring of human exposure to estrogens has historically focused on exogenous environmental and dietary estrogens (Artacho-Cordon et al., 2017; Asimakopoulos et al., 2012; Azzouz et al., 2016; Dufour et al., 2016; Hines et al., 2015; Marks et al., 2017; Shekhar et al., 2017; Todaka et al., 2005; Wan et al., 2013). Although endogenous estrogens such as E1, E2, E3 and E4 are an arguably equal or more important source of estrogenicity, concomitant measurement of these estrogens in biomonitoring studies is uncommon. This bias in estrogen biomonitoring leads to an important deficit in the understanding of estrogenicity, specifically in the characterization of normal estrogenicity and what constitutes disruption of normal estrogenicity. In the biological context of the complex mixture of multiple circulating estrogens in humans, the bioavailable concentrations of these hormones establish the baseline estrogenicity upon which the dietary and environmental estrogens must compete. The premise of our study was that the contribution of exogenous estrogens should be benchmarked against this normal, but variable background in serum estrogenicity (Fleck et al., 2018). The premise is based on a strong biological argument, clear evidence of normal variability in estrogen levels and anchored in current clinical practice.

Within-person variability in estrogen levels over short periods of hours or minutes is consistent with a tolerance to normal biological variation in serum estrogens. For example, we observed within-person variability in concentrations of E1, E2, and E3 of 5.6–27.2%, 4.6–16.1% and 5.5–15.6% respectively (Fleck et al., 2018). This confirms and extends much earlier work that showed similar within-person variability in E1 and E3 concentrations over the course of measurements taken 30 min to several hours apart. Summarizing the work of other authors, Oakey reported diurnal variations in unconjugated E3 of 20–40% and 30–70% in two 8-person cohorts (Oakey, 1979). For E2, the individual maximum coefficient of variation was 14% (Oakey, 1979). In another example, variability, measured as multiple (as many as eight) peak concentrations of E2, was reported in a study of 22 healthy pregnant women in the third trimester when serial samples were taken every thirty minutes (Magiakou et al., 1996b). Reck and co-workers reported variations from the mean values of 3 pregnant women of up to 40%

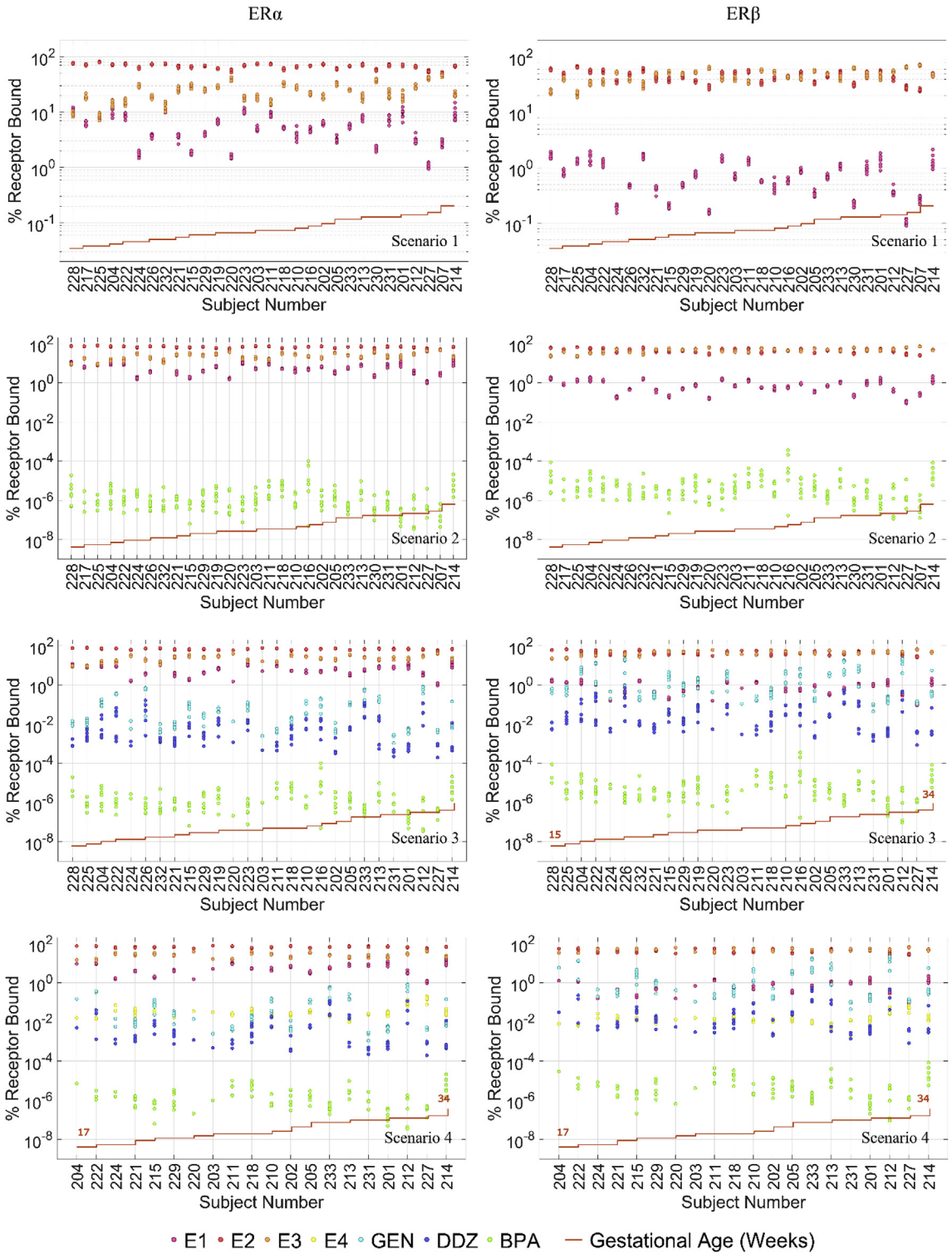

Fig. 3. The calculated percent of ERα (left panel) and ERβ (right panel) receptors occupied by the seven measured estrogens. Each point represent calculations made for up to seven measurements made in each member of the cohort over a day. Scenarios 1–4 are presented in order from the top to bottom.

across serial samples taken between thirty minutes and one hour apart over a 24 h period (Reck et al., 1979). The level of normal variability of serum estrogens over durations as short as thirty minutes in pregnant and non-pregnant women consistently reported in these studies is strong evidence of a normal, acceptable level of variability in estrogens of around 30% (some higher, some lower). It should also not be lost that the time-scale of these normal fluctuations is similar to those resulting from episodic exposures to phytoestrogens like GEN and DDZ and synthetic estrogens like BPA, which are ingested principally through the diet. Overall, the large inter-individual variability in normal estrogen concentrations reported during the menstrual cycle and pregnancy implies wide latitude for biologically normal concentrations, and consequently, correspondingly significant inter-individual differences in total serum estrogenicity. Variability in the dominant systemic estrogens has not been considered outside the clinical endocrinology community. Efforts to establish norms for what constitutes unacceptable

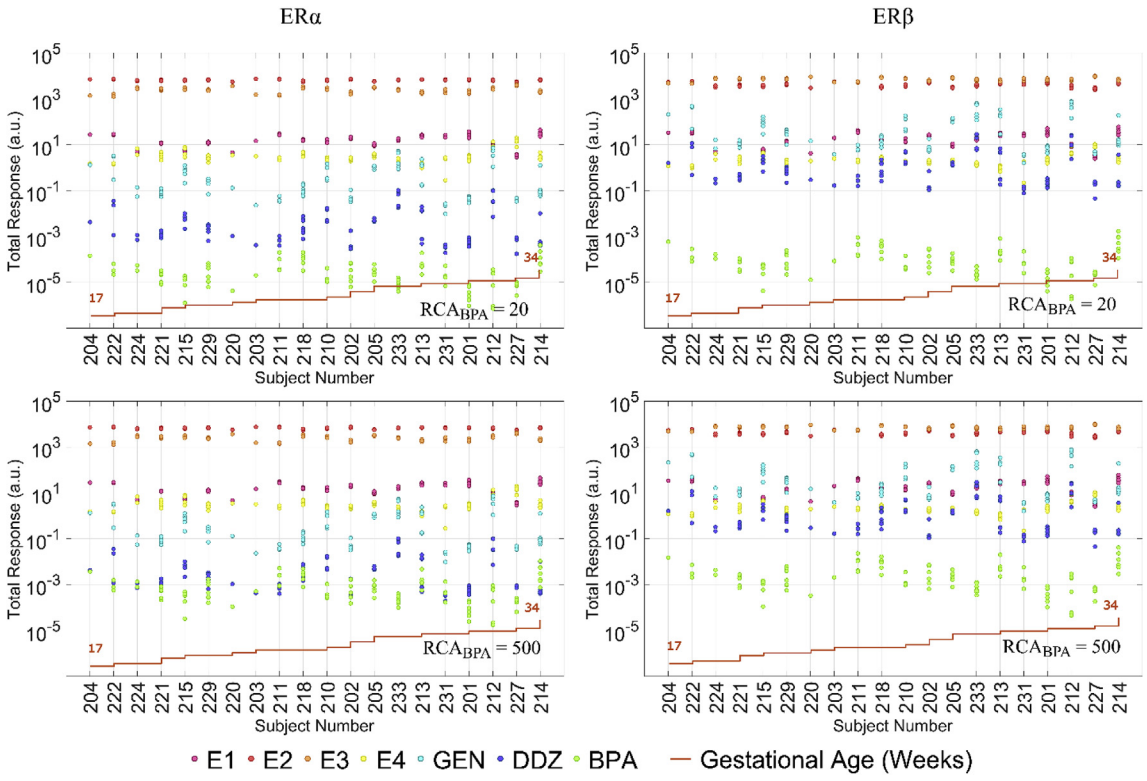

Fig. 4. The calculated total estrogenic response through ER-α (left panel) and ER-β (right panel) receptors occupied by the seven measured estrogens for Scenario 4. Each point represent calculations made for up to seven measurements made in each member of the cohort over a day.

Table 5
Percent receptor occupancy of various ligands and proteins in different phases of menstrual cycle.

Estrogen	ER-α			ER-β		
	Phase of Menstrual Cycle					
	Follicular	Ovulatory	Luteal	Follicular	Ovulatory	Luteal
E1	5.3	12	5.6	0.52	1.9	0.74
E2	58	76	77	29	62	51
E3	3.6	0.79	0.61	5.9	2.1	1.4
TOTAL	67	89	83	35	66	54

Table 6
Concentration in nM of various ligands and proteins in different phases of menstrual cycle.[a]

Ligand	Phase of the Menstrual Cycle		
	Follicular	Ovulatory	Luteal
E1	0.23	1.6[b]	0.46
E2	0.29	1.2[c]	0.72
E3	0.10	0.066[d]	0.032
TES	1.3	1.6[e]	1.3
Protein			
SHBG	37	37[f]	37
Albumin	560000	560000[f]	560000

[a] Taken from Dunn et al. (1981) unless noted otherwise.
[b] Obtained as × 7 of the concentration of follicular phase as evidenced by data presented in Reed and Carr (2015).
[c] Converted to nM from pg/ml value reported in Chatzidimitriou et al. (2015).
[d] Assumed to be the average of follicular and luteal phases.
[e] Obtained as × 1.2 of the concentration of follicular phase based on data from (Reed and Carr, 2015).
[f] Assumed to be the same as the follicular and luteal phases due to the absence of data.

excursions from normal serum estrogenicity, a possible marker for risk of endocrine disruption, for example by researchers and regulatory agencies have not considered endogenous estrogens. This perspective has influenced both test system design for screening environmental estrogens and key research hypotheses.

A widely promoted hypothesis in environmental health is that exposure to very small amounts (no specific definition) of synthetic estrogens can have profound disruptive effects (Welshons et al., 2003), even in the face of normal, variable levels of biologically active endogenous estrogens. An underlying assumption of this belief is that estrogenicity is nearly constant and strictly controlled and that small deviations in estrogen exposure or estrogenicity can irreversibly disrupt normal biology. As we have pointed out (Fleck et al., 2018), clinical judgements regarding serum estrogenicity contrast sharply with this view. Measurement of estrogen concentrations in serum and calculation of bioavailable hormone fractions are common practice in clinical endocrinology (Belgorosky et al., 1987; Belgorosky and Rivarola, 1988; Cauley et al., 1999; Egleston et al., 2010; Faix, 2013; Hackbarth et al., 2011; Santen et al., 2015; Sartorius et al., 2009; Shea et al., 2014). Clinical assessments are conducted by comparing measured serum estrogen levels to published reference ranges to assess the potential for health impact related to abnormal levels of estrogens. In other words, consideration of normal variability, captured in reference ranges, is the norm (Mayo, 2018). The assessment of pregnancy (Demers et al., 2015; Gerhard and Runnebaum, 1984; Pillai et al., 2016), fertility (Behre et al., 2000; Demers et al., 2015; Smith et al., 1980), and developmental status (Demers et al., 2015) are examples where such comparisons form the basis of clinical diagnoses. Published reference ranges used in these assessments reflect the normal variation in estrogen levels in women throughout the day, the phases of the menstrual cycle, by life stage, during pregnancy, and between individuals (Abbassi-Ghanavati et al.,

Table 7
Receptor percent occupancy summary statistics for scenarios 1-4.

Scenario	Estrogen	ER-alpha				ER-beta			
		Median	Min	Max	IQR	Median	Min	Max	IQR
1	E1	5.4	0.94	15	5.1	0.67	9.1E-02	2.1	0.86
	E2	69	48	81	10	45	24	69	17
	E3	22	7.0	48	12	46	19	74	15
	TOTAL*	**97**	**94**	**99**	**1.8**	**93**	**83**	**98**	**4.4**
2	E1	5.5	0.95	15	5.3	0.68	9.0E-02	2.1	0.84
	E2	68	48	81	10	45	25	68	17
	E3	23	7.8	47	12	47	20	72	15
	BPA	9.1E-07	3.5E-08	1.0E-04	2.0E-06	3.7E-06	1.2E-07	3.8E-04	8.0E-06
	TOTAL*	**97**	**94**	**99**	**1.8**	**93**	**83**	**98**	**4.2**
3	E1	6.3	0.96	15	5.0	0.76	9.1E-02	2.1	0.78
	E2	68	51	81	7.3	44	25	67	16
	E3	22	7.9	45	10	44	21	68	14
	GEN	2.5E-02	1.1E-03	0.82	0.11	0.98	4.5E-02	22	3.7
	DDZ	2.6E-03	2.0E-04	0.16	1.1E-02	1.6E-02	8.4E-04	0.73	6.7E-02
	BPA	9.9E-07	3.5E-08	1.0E-04	2.1E-06	3.8E-06	9.1E-08	3.6E-04	8.2E-06
	TOTAL*	**97**	**94**	**99**	**1.8**	**93**	**83**	**98**	**4.3**
4	E1	5.3	0.96	15	4.8	0.57	9.0E-02	2.1	0.70
	E2	67	50	76	7.8	42	25	59	15
	E3	25	13	45	11	48	31	68	11
	E4	2.8E-02	2.8E-03	0.20	1.6E-02	1.3E-02	1.5E-03	7.0E-02	5.8E-03
	GEN	1.8E-02	1.1E-03	0.82	0.11	0.59	4.4E-02	22	3.4
	DDZ	1.8E-03	2.0E-04	0.12	7.5E-03	9.6E-03	8.4E-04	0.54	3.5E-02
	BPA	1.1E-06	3.5E-08	2.1E-05	2.3E-06	3.8E-06	9.1E-08	8.3E-05	8.2E-06
	TOTAL*	**98**	**94**	**99**	**1.6**	**94**	**88**	**98**	**3.5**

* The summary statistics corresponding to TOTAL refers to the statistics for the total receptor percent occupancy, i.e. the sum of percent receptor occupancies of all the ligands in each scenario.

Table 8
Total response (a.u.) summary statistics.

Scenario	Estrogen	ER-alpha				ER-beta			
		Median	Min	Max	IQR	Median	Min	Max	IQR
1	E1	16	2.8	44	15	19	2.5	60	24
	E2	6.9E+03	4.8E+03	8.1E+03	1.0E+03	4.5E+03	2.4E+03	6.9E+03	1.7E+03
	E3	2.1E+03	6.7E+02	4.6E+03	1.1E+03	6.9E+03	2.8E+03	1.1E+04	2.2E+03
	TOTAL*	**9.1E + 03**	**8.3E + 03**	**9.5E + 03**	**4.0E + 02**	**1.2E + 04**	**9.3E + 03**	**1.3E + 04**	**1.0E + 03**
RCA for BPA = 20									
4	E1	16	2.9	45	14	16	2.5	60	20
	E2	6.7E+03	5.0E+03	7.6E+03	7.8E+02	4.2E+03	2.5E+03	5.9E+03	1.5E+03
	E3	2.4E+03	1.2E+03	4.3E+03	1.1E+03	7.1E+03	4.7E+03	1.0E+04	1.6E+03
	E4	2.8	0.28	20	1.6	2.0	0.22	10	0.86
	GEN	0.17	9.8E-03	7.5	1.0	21	1.6	7.9E+02	1.2E+02
	DDZ	1.6E-03	1.8E-04	0.10	6.6E-03	0.52	0.05	29	1.9
	BPA	2.2E-05	7.0E-07	4.2E-04	4.5E-05	7.6E-05	1.82E-06	1.7E-03	1.6E-04
	TOTAL*	**9.1E + 03**	**8.3E + 03**	**9.4E + 03**	**2.6E + 02**	**1.2E + 04**	**1.0E + 04**	**1.3E + 04**	**5.7E + 02**
RCA for BPA = 500									
4	E1	16	2.9	45	14	16	2.5	60	20
	E2	6.7E+03	5.0E+03	7.6E+03	7.8E+02	4.2E+03	2.5E+03	5.9E+03	1.5E+03
	E3	2.4E+03	1.2E+03	4.3E+03	1.1E+03	7.1E+03	4.7E+03	1.0E+04	1.6E+03
	E4	2.8	0.28	20	1.6	2.0	0.22	10	0.86
	GEN	0.17	9.8E-03	7.5	1.0	21	1.6	7.9E+02	1.2E+02
	DDZ	1.6E-03	1.8E-04	0.10	6.6E-03	0.52	0.05	29	1.9
	BPA	5.5E-04	1.7E-05	1.1E-02	1.1E-03	1.9E-03	4.6E-05	4.2E-02	4.1E-03
	TOTAL*	**9.1E + 03**	**8.3E + 03**	**9.4E + 03**	**2.6E + 02**	**1.2E + 04**	**1.0E + 04**	**1.3E + 04**	**5.7E + 02**

* The summary statistics corresponding to TOTAL refers to the statistics for the total response, i.e. the sum of individual responses of all the ligands in each scenario.

2009; Blackwell et al., 2013; Katagiri et al., 1976; Magiakou et al., 1996a; Oakey, 1979; Panico et al., 1990; Ray et al., 2012; Reck et al., 1979; Rothman et al., 2011). Thus in clinical practice, some variability in biologically active hormones is considered inherently normal, and not an indication of clinically relevant disruption of estrogen signaling or an association with adverse effects.

We know of no formally accepted guidelines for determining the level of excursion from normally variable levels of estrogens sufficient to disrupt normal biology. For purposes of this analysis we proposed a criteria based on a minimal increment of normal variation: $1/1000^{th}$ (0.1%) of normal daily variability in estrogenicity. Beyond the analysis here, establishment of formal criteria for purposes of risk assessment would necessarily involve scientific bodies and/or regulatory agencies. Our hypothesis was that synthetic and/or dietary estrogens must contribute sufficient "added" estrogenicity to shift total estrogenicity during pregnancy by a meaningful increment of normal intra-individual daily variability to be considered abnormal. In order to explore our hypothesis, we measured concentrations of E1, E2, E3, E4, DDZ, GEN, and BPA in a thirty member cohort of pregnant women (Fleck et al., 2016, 2018; Teeguarden et al., 2016). We next calculated the comparative estrogenic potential for each chemical and compared them to the values of FRO and TR that would reflect biological meaningful increments of normal variability based on our criteria.

Based on calculated FROs and TRs, the contribution of BPA to total estrogenicity was several orders of magnitude lower than the $1/1000^{th}$ of the measured variability in our cohort criteria for biological meaningful contributions to total estrogenicity (Table 7, Table 8). This was the case even when we assumed the RCA for BPA was five times greater than E2. Importantly, measured serum concentrations of GEN and DDZ always exceeded our criteria in the case of GEN, by two orders of magnitude for ERβ. Two clear implications of these analyses are that unless BPA is simultaneously a structural analogue of estrogen similar to GEN and DDZ but has a truly unique RCA or overall relative potency for ER-α or ER-β, that are orders of magnitude higher than the other structural analogues, it is implausible that BPA disrupts normal biology through ERα or ERβ during pregnancy in women selected for higher potential exposure to BPA. Secondly, when extrinsic and intrinsic properties—exposure levels, pharmacokinetics, receptor affinities and RCAs—of the dietary and environmental estrogens are collectively considered, GEN and DDZ individually and collectively contribute far more estrogenicity than BPA in this cohort of pregnant women.

Hypotheses regarding the estrogenicity of dietary and synthetic estrogens in the context of physiologically normal and variable endogenous hormones could be extensively tested in vitro, but have not. The results of screening studies conducted under more standard conditions where endogenous hormones are not present (Conley et al., 2016; Dvorakova et al., 2016; Judson et al., 2015; Kunz et al., 2017; OECD; Silva et al., 2002) are not immediately informative about the impact of the added estrogenicity in intact systems such as humans. In these intact systems, multiple biologically active endogenous estrogens such as E1-E4, the presence of metabolic and excretory function, and serum protein binding systems, all contribute significantly to the overall estrogenic potential (Conley et al., 2016; Nagel et al., 1998). We hypothesize that re-evaluation of several classes of environmental hormones—estrogens, androgens—using in vitro systems adapted to better reflect proper physiological context would provide more immediately interpretable hazard data with greater potential for impact on public health and regulation. Moreover, the biological mechanisms identified, especially where real-world exposure levels were considered, would be of greater biological relevance than similar studies conducted in systems operating outside of a more representative physiological context. We also point out that similar consideration for two key differences in human and rodent physiology should be considered when extrapolating findings from rodents to humans. Specifically, rodents have much lower circulating levels of estrogens (Nilsson et al., 2015) and SHBG is absent in adult rodents, potentially influencing bioavailable levels of estrogens.

Several assumptions were made in our analyses to overcome limitations in data or missing information. In the absence of RCA values for E4, we assumed that the RCA for E4 was closest to the most structurally similar analogue, E3. For BPA, we elected to assign two RCA values. One RCA was within the bounds of RCA values reported for structural analogues E1-E3, GEN and DDZ (Jeyakumar et al., 2011; Jiang et al., 2013) for ER-α and ER-β. These values are informative about the general magnitude of differences in RCA across a structurally diverse array of estrogens. The data from Jayakumar and co-workers are consistent with bounds for the intrinsic factors influencing relative potency of estrogens being between 0.1% and no more that 200%. We elected to use these approximate bounds to explore the impact of RCA on the contribution of BPA to total serum estrogenicity in our cohort. Thus, a second upper bound RCA value for BPA of five times the value of E2 was chosen. Even under these conditions, the influence of BPA was *de minimus*, a conclusion that would be unaltered even if the RCA for BPA was several orders of magnitude higher than we assumed, which even if theoretically possible, is not biologically likely, as evidenced by the published data for an array of estrogenic compounds.

Apart from the specific analyses presented in this study, the overall approach developed here should be seen as a generalized framework for calculating the total and relative activity of any measurable series of ligands with tissue receptors from serum biomonitoring data. In contrast to experimental systems for assaying total hormone activity (e.g. estrogenicity), like the CULEX, the framework developed here allows attribution of relative estrogenicity to each measurable ligand. The three elements of the framework—biomonitoring, calculation of bioavailable concentrations, and solving multiple ligand-receptor equilibria to estimate receptor binding—are both robust and well-recognized in the clinical research community. They are also extendable to any measurable ligand-receptor systems, such as androgens, and dioxins. The required data for calculating the total and relative hormonal activity are obtainable through routine experiments, receptor affinity assays for example, although measures of RCA are far more challenging to obtain experimentally. Nonetheless, development of a library of receptor affinities, binding constants for serum proteins that influence bioavailable concentrations alone would extend the capability here to many other ligand-receptor systems of importance to public health. Adaptation of the simulation code developed here to these systems would allow rapid assessment of the relative importance of any biologically active environmental compounds to such receptor systems. With the rapid expansion of biomonitoring driven by pursuit of the exposome, frameworks for deriving understanding about the biological implications of these exposures will only increase in importance.

5. Conclusions

The framework developed here for assessing the relative importance of endogenous, dietary, and environmental estrogens from biomonitoring data can be extended for application to any ligand-receptor system. Applied to a group of seven estrogens measured in the serum of 30 pregnant women, we found that the contribution of BPA to total serum estrogenicity were, based on the two metrics of estrogenicity used in this study, namely, the FRO and TR, multiple orders of magnitude less than the estrogenicity of E1, E2 and E3, even under conditions where BPA was assumed to be five times more potent than endogenous E2. In contrast, estrogenicity associated with measured serum concentrations of GEN and DDZ was intermediate, based on higher serum concentrations and receptor affinity than BPA.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fct.2018.12.013.

Transparency document

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