Science of the Total Environment

Developmental Programming: Impact of preconceptional and gestational exposure to a real-life environmental chemical mixture on maternal steroid, cytokine and oxidative stress milieus in sheep.

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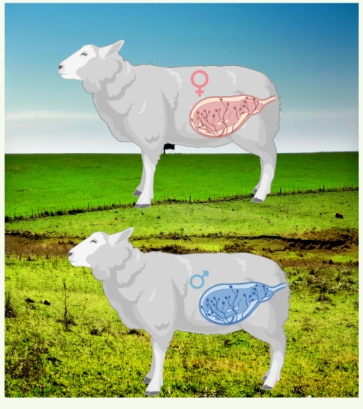
Abstract:	Background: Gestational exposure to environmental chemicals (ECs) is associated with adverse, sex-specific offspring health effects of global concern. As the maternal steroid, cytokine and oxidative stress milieus can have critical effects on pregnancy outcomes and the programming of diseases in offspring, it is important to study the impact of real-life EC exposure, i.e., chronic low levels of mixtures of ECs on these milieus. Sheep exposed to biosolids, derived from human waste, is an impactful model representing the ECs humans are exposed to in real-life. Offspring of sheep grazed on biosolids-treated pasture are characterized by reproductive and metabolic disruptions. Objective: To determine if biosolids exposure disrupts the maternal steroid, cytokine and oxidative stress milieus, in a fetal sex-specific manner. Methods: Ewes were maintained before mating and through gestation on pastures fertilized with biosolids (BTP), or inorganic fertilizer (Control). From maternal plasma collected mid-gestation, 19 steroids, 14 cytokines, 6 oxidative stress markers were quantified. Unpaired t-test and ANOVA were used to test for differences between control and BTP groups (n=15/group) and between groups based on fetal sex, respectively. Correlation between the different markers was assessed by Spearman correlation. Results: Concentrations of the mineralocorticoids - deoxycorticosterone, corticosterone, the glucocorticoids - deoxycortisol, cortisol, cortisone, the sex steroids - androstenedione, dehydroepiandrosterone, 16-OH-progesterone and reactive oxygen metabolites were higher in the BTP ewes compared to Controls, while the proinflammatory cytokines IL-1β and IL-17A and anti-inflammatory IL-36RA were decreased in the BTP group. BTP ewes with a female fetus had lower levels of IP-10. Discussion: These findings suggest that pre-conceptional and gestational exposure to ECs in biosolids increases steroids, reactive oxygen metabolites and disrupts cytokines in maternal circulation, likely contributors to the aberrant phenotypic outcomes seen in offspring of BTP sheep - a translationally relevant precocial model.
Response to Reviewers:	

Graphical Abstract

Maternal Biosolids Exposure

Biosolids applied to pastures

Sheep raised on biosolids treated pastures



Changes in Maternal Plasma at Mid-gestation

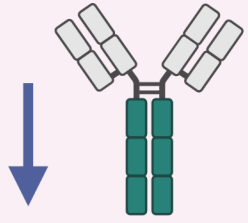
Steroid hormones

Deoxycorticosterone, Corticosterone, Deoxycortisol, Cortisone, Cortisol, 16-OH-Progesterone, Androstenedione, DHEA

Oxidative Stress

Reactive oxygen metabolites

Cytokines

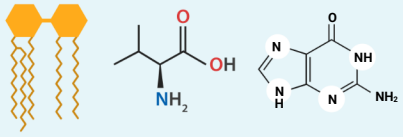


IL-1β, IL-17A, IL-36RA

Phenotypic Changes Reported Previously

Maternal Milieu

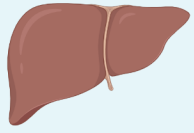
Disrupted maternal metabolome



Offspring Phenotype and Function

Bone mineral density

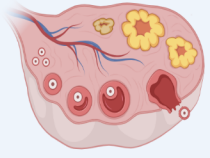
Hepatic gene & protein expression



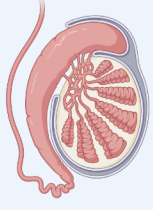
Reproductive axis

Neuroendocrine

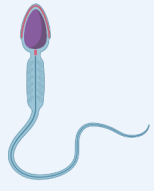
Ovary



Testis



Sperm



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Highlights:

- Gestational exposure to biosolids disrupts maternal circulatory milieus in sheep.
- Biosolids exposed sheep is an impactful model of real-life EC exposures in humans.
- Biosolids disrupts mid-gestational steroid, cytokine and oxidative-stress status.
- Biosolids perturbs dialogue amid maternal steroids, cytokines and oxidative stress.
- Biosolids-induced maternal disruptions may contribute to adverse offspring outcomes.

Developmental Programming: Impact of preconceptional and gestational exposure to a real-life environmental chemical mixture on maternal steroid, cytokine and oxidative stress milieus in sheep.

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Abstract:

Background: Gestational exposure to environmental chemicals (ECs) is associated with adverse, sex-specific offspring health effects of global concern. As the maternal steroid, cytokine and oxidative stress milieus can have critical effects on pregnancy outcomes and the programming of diseases in offspring, it is important to study the impact of real-life EC exposure, i.e., chronic low levels of mixtures of ECs on these milieus. Sheep exposed to biosolids, derived from human waste, is an impactful model representing the ECs humans are exposed to in real-life. Offspring of sheep grazed on biosolids-treated pasture are characterized by reproductive and metabolic disruptions.

Objective: To determine if biosolids exposure disrupts the maternal steroid, cytokine and oxidative stress milieus, in a fetal sex-specific manner.

Methods: Ewes were maintained before mating and through gestation on pastures fertilized with biosolids (BTP), or inorganic fertilizer (Control). From maternal plasma collected mid-gestation, 19 steroids, 14 cytokines, 6 oxidative stress markers were quantified. Unpaired t-test and ANOVA were used to test for differences between control and BTP groups (n=15/group) and between groups based on fetal sex, respectively. Correlation between the different markers was assessed by Spearman correlation.

Results: Concentrations of the mineralocorticoids - deoxycorticosterone, corticosterone, the glucocorticoids - deoxycortisol, cortisol, cortisone, the sex steroids - androstenedione, dehydroepiandrosterone, 16-OH-progesterone and reactive oxygen metabolites were higher in the BTP ewes compared to Controls, while the proinflammatory cytokines IL-1β and IL-17A and anti-inflammatory IL-36RA were decreased in the BTP group. BTP ewes with a female fetus had lower levels of IP-10.

Discussion: These findings suggest that pre-conceptional and gestational exposure to ECs in biosolids increases steroids, reactive oxygen metabolites and disrupts cytokines in maternal circulation, likely

contributors to the aberrant phenotypic outcomes seen in offspring of BTP sheep - a translationally relevant precocial model.

Keywords:
Prenatal exposure, biosolids, maternal physiology, steroids, cytokines, oxidative stress

1. Introduction:

Non-communicable diseases are collectively responsible for 74% of deaths worldwide [1] and pose a huge economic burden [2], especially in low and middle-income countries [3]. Humans are chronically exposed to a wide variety of environmental chemicals (ECs) in our everyday life and gestational exposure to ECs like bisphenol A, triclosan, phthalates, paraben and persistent chemicals can adversely affect pregnancy outcomes [4] and offspring health [5-7]. Gestational exposure to ECs plays an important role in the developmental origin of diseases [8] including type 2 diabetes [9], cardiovascular disease [10,11], and reproductive disorders [12,13] due to effects on the endocrine [14], reproductive [13,15], immune [16], metabolic [17-19], and nervous [20,21] systems.

While ECs can have direct effects on offspring development a number of studies report effects of EC exposure on the maternal physiology [22,23] and their potential effects on markers of offspring health [24,25]. These studies mainly addressed the impact of individual chemicals or mixtures of a few limited chemical classes, but this does not reflect real-life EC exposure which entails chronic exposure to low levels of mixtures of several classes of ECs. Exposure to a mixture of chemicals can have additive, synergistic or antagonistic effects [26,27], with different biological consequences than single chemical exposure [28]. It's not possible to formulate a chemical mixture that truly represents human EC exposure as each individual's exposure pattern and dosage will differ. However, there is a need to study the effects of exposure paradigms that more closely reflect the human exposome. Biosolids, produced from

domestic waste-water treatment processing (sewage sludge) are used widely in agriculture as an alternative to inorganic fertilizers [29]. Due to their anthropogenic nature, biosolids represent the array and concentrations of chemicals that humans are exposed to in real-life, including those with endocrine disrupting potential such as perfluoroalkyl substances (PFAS) and bisphenol A [30-32]. Previous studies using sheep exposed to BTP during gestation have demonstrated accumulation of endocrine disrupting chemicals in both adult and fetal tissues [33]. Thus, sheep grazed on biosolids treated pastures (BTP) provide a novel experimental and a proof-of-concept model to investigate the adverse effect of exposure to real-life EC mixtures, on the maternal hormonal and metabolic milieu that influence the developmental environment of the fetus.

Exposure to various ECs can impact the maternal metabolic [34,35], steroidal [36], inflammatory cytokine [37] and oxidative stress [38,39] milieus, all key players in the maintenance of maternal homeostasis and fetal development. Steroids are programming agents [40-42], which play an important role in directing fetal growth [43,44]. It has been demonstrated that various ECs can perturb the steroid milieu [45] or function as steroid mimics [46,47]. A delicate balance amongst messengers of the maternal immune system, the pro- and anti-inflammatory cytokines [48], is required for the maintenance of a healthy pregnancy [49,50]. In humans, exposure to persistent ECs [51] and chemical mixtures [52] is associated with perturbations in the maternal cytokine milieu. Several ECs like PBDEs, PFAS, BPA have been shown to produce a maternal inflammatory response [51,53], which can compromise pregnancy outcomes [54] as well as the long-term health of offspring [55-58]. Oxidative stress is another important maternal contributor to healthy pregnancies [50,59] and offspring outcomes [60], as increased oxidative stress perturbs nutrient transport and oxygen supply to the developing fetus, leading not only to adverse pregnancy but also offspring pathologies [61]. It is also a key cellular response to EC exposure [62], as human studies have revealed an

association between EC exposure and increased maternal oxidative stress [38,63] with animal studies providing causal links [64].

Fetal sex influences the maternal-placental-fetal interaction, is associated with maternal hormone systems [65] and maternal outcomes [66,67] and is also a critical factor in influencing maternal response to EC exposures [23,36]. The impact of fetal sex on the maternal environment can be exerted at the level of steroids [68,69], inflammatory mediators [70-72] and oxidative stress markers [38,73], which are all developmental risk factors associated with EC exposures.

Considerable interrelationships exist between steroidogenic, inflammatory, and oxidative stress pathways with each having the potential to influence the other. Pregnancy is associated with an anti-inflammatory phenotype with the increase in levels of estrogen, progesterone and glucocorticoids mediating the immunological changes in pregnancy [74]. Several studies also provide evidence relative to the impact of the steroid hormones on the inflammatory markers [75-78]. A close relationship also exists between inflammatory and oxidative stress cascades during pregnancy and an association between inflammatory and oxidative stress markers has been evidenced in relation to various EC exposure in cohort studies. [38,53]. Although several studies have established that maternal exposure to ECs are associated with increased maternal steroids, inflammatory cytokines and oxidative stress markers [36,38,52], their role in modulating the inter-relationship between these milieus warrants investigation due to the importance of the steroidal, inflammatory, and oxidative stress milieus in determining pregnancy and fetal outcomes.

Overall, while considerable evidence exists regarding the impact of individual ECs, or a class of ECs, in perturbing the maternal steroidal, inflammatory and oxidative stress milieu, a gap remains as to how this translates to the real-life exposure to mixtures of ECs. This is important as these key maternal mediators of fetal growth and differentiation underpin the developmental origins of disease. The current

study addresses this void by testing the hypothesis that exposure to biosolids, a real-life EC mixture resource relevant to human exposure, perturbs the maternal steroid, oxidative and inflammatory milieus, and their inter-relationship at multiple levels; and that these are altered depending on the sex of the fetus. In so doing, this study builds on the findings reported previously[34] that EC exposure in this model leads to profound alterations in maternal metabolism, including perturbations to lipid, amino acid and one carbon metabolism consistent with the aforementioned adverse phenotypic outcomes in offspring.

2. Methods:

2.1. Ethics statement: The animals were maintained at the University of Glasgow Cochno Farm and Research Centre under normal husbandry conditions and the procedures were conducted in accordance with the UK Home Office Animals (Scientific Procedures) Act 1986 regulations under license (PPL PF10145DF).

2.2. Animals: EasyCare ewes (*Ovis aries*) were maintained as described in detail earlier [34] on pastures fertilized with conventional rates of biosolids (BTP) (4 tonnes/ha, twice per annum) or inorganic fertilizer (Control), with both pastures supplied with 225 kg N/ha per annum. Duration of exposure began 4 weeks prior to mating and lasted through the duration of pregnancy. Ewes were randomly allocated to either the Control or BTP. Semen from four rams maintained on Control pastures was used for laparoscopic artificial insemination (AB Europe, Edinburgh UK) of both Control and BTP ewes. Plasma from gestational day 90 (term: 147-days) pregnant ewes (Control, n = 15; BTP, n =15) were shipped on dry ice to the University of Michigan where it was stored at -80 °C until analysis. Ewes pregnant with mixed sex fetuses were excluded from the fetal sex-specific analyses. The animals and

plasma samples used in the current study are the same as that used in the previously published untargeted metabolomics study [34].

2.3. Steroid measurements: Circulating steroids were quantified in plasma samples with the aid of Agilent 6495 triple quadrupole mass spectrometer and Agilent 1260 and 1290 dual front-end HPLC/UPLC via liquid chromatography-tandem mass spectrometry processes [36,79,80]. Briefly, plasma (0.1 mL) was combined with internal standards, diluted with water, and loaded onto a supported liquid extraction cartridge (Isolute, Biotage, Charlotte, NC). Steroids were eluted twice with 0.7 mL methyl-*tert*-butyl ether, dried under vacuum (Savant, Thermo Fisher), and reconstituted in 0.1 mL 40:60 methanol: water. The steroids measured included 24 Δ4 steroids: cortisol, cortisone, corticosterone, 11-deoxycortisol, 11-deoxycorticosterone, 18-OH-cortisol, 11-OH-androstenedione, androstenedione, 11-OH-progesterone, 16-OH-progesterone, 17-OH-progesterone, progesterone, 18-OH-corticosterone, 18-oxocortisol, 21-deoxycortisol, aldosterone, testosterone, estradiol, estrone, estriol, 11-OH-testosterone, 11-ketoandrostenedione, 11-ketoprogesterone, 11-ketotestosterone and 7 Δ5- or 5α-reduced steroids: 17-OH-pregnenolone, 17-OH-allopregnanolone, dehydroepiandrosterone (DHEA), androsterone, pregnenolone, allopregnanolone and androstenediol. The lower limit of quantitation (LLOQ) for each analyte was estimated from the signal-to-noise ratio of pooled samples using Mass Hunter 'peak to peak' method. Review of extracted ion chromatograms confirmed that all samples with values reported above the LLOQ gave reliably quantifiable peaks at the correct retention time. The inter and intra-assay coefficients of variation were <12% for each analyte. The steroid variables were log-transformed, tested for normality using Kolmogorov-Smirnov test, and values below LLOQ were set at their respective LLOQ levels as indicated in Table 1.

2.4. Measurement of oxidative stress markers: Plasma reactive oxygen metabolites (ROM) and total antioxidant status (TAS) were measured using manufacturer's instructions on commercial test kits (Rel Assay Diagnostics, Turkey) that are based on the colorimetric method developed by Erel [81,82]. Malondialdehyde (MDA) concentrations were determined with thiobarbituric acid using commercial TBARS assay kit (Cayman Chemicals, Ann Arbor, MI) based on Esterbauer and Cheeseman procedure [83]. The oxidized amino acids – chlorotyrosine, nitrotyrosine and dityrosine were quantified by liquid chromatography (LC)-electrospray ionization tandem mass spectrometry (MS/MS) with multiple reaction monitoring MS/MS positive ion acquisition mode using an Agilent 6410 triple quadrupole MS system equipped with an Agilent 1200 LC system (Agilent Technologies, Santa Clara, CA) as described earlier [84]. The concentrations of chlorotyrosine, nitrotyrosine and dityrosine were normalized for total tyrosine and expressed as the ratio of the oxidized product over the total tyrosine (μM/mol of Y). The intra-assay coefficient of variation for chlorotyrosine, nitrotyrosine and dityrosine were 4.7%, 14.6% and 5.8% respectively, and the detection limit was 0.0001 μM/mol tyrosine for all of the oxidized amino acids.

2.5. Measurement of cytokines: The concentrations of 14 cytokines –interleukin -1 alpha (IL-1α), interleukin -1 beta (IL-1β), interleukin -4 (IL-4), interleukin -6 (IL-6), interleukin -10 (IL-10), interleukin -17A (IL-17A), interleukin -36 receptor antagonist (IL-36RA), macrophage inflammatory protein-1 alpha (MIP-1α), macrophage inflammatory protein-1 beta (MIP-1β), interferon -gamma (IFN-γ), interferon-gamma inducible protein- 10 (IP-10), tumor necrosis factor- alpha (TNF-α) and vascular endothelial growth factor -A (VEGF-A), were quantified in maternal plasma simultaneously using MILLIPLEX® MAP Ovine Cytokine/Chemokine Panel-1 96-well plate assay (SCYT1-91K, Merck Millipore, Boston, MA), according to the manufacturer's instructions. The cytokine concentrations were

expressed as pg/mL. The intra-assay coefficients of variation were <20% for each cytokine. The cytokine concentrations below limit of detection (LOD) were replaced with LOD/$\sqrt{2}$.

2.6. Correlation Analysis: The Spearman correlations between the concentrations of steroid hormones, oxidative stress markers and cytokines were analyzed separately for the Control, BTP, Control-Male, BTP-Male, Control-Female and BTP- Female groups and visualized using the corrplot *R* package.

2.7. Statistical analyses: Steroid, oxidative stress marker and cytokine concentrations were log-transformed; outliers were removed based on ROUT method and data was tested for normality using Kolmogorov-Smirnov test, for each analysis. The analyses were conducted both for the full cohort, and on stratification of animals based on fetal-sex to assess the fetal sex-specific effects of biosolids exposure. Statistical significance was assessed by unpaired t-test for difference between Control and BTP groups. Two-way ANOVA testing fetal sex effects included only ewes carrying same sex twins (Control - male fetus = 9, female fetus = 5; BTP- male fetus = 5, female fetus = 7). Multiple comparisons were corrected using Tukey's post hoc test. P values < 0.05 were considered significant. In addition, magnitude of difference between Control and BTP groups was estimated using Cohen's effect size analysis with Cohen's d value between 0.5 and 0.8 representing 'medium', and Cohen's d value >0.8 representing 'large' magnitude differences. A large Cohen's d indicates the mean difference is large compared to the variability and the impact is significant in real-world scenarios, a medium effect size indicates a reasonable overall impact and a small effect size represents negligible differences between the groups [85]. Graphs were generated using GraphPad Prism version 9.5.0 (GraphPad Software, CA, USA).

3. Results:

3.1. Effect of biosolids exposure on steroids in maternal plasma: The mean concentrations of twelve Δ4 steroids and seven Δ5- or Δ5α-reduced steroids detected in the plasma of Control and BTP ewes are listed in Table 1. Compared to Control ewes, BTP ewes had significantly higher circulating concentrations of the mineralocorticoids (deoxycorticosterone and corticosterone) and the glucocorticoids (deoxycortisol, cortisol and the cortisol metabolite, cortisone) (Figure 1). BTP ewes also had higher circulating concentrations of the sex steroids androstenedione, dehydroepiandrosterone and 16-OH-progesterone (Figure 1). The concentrations of 18-OH-cortisol and 17-OH-progesterone were higher in the BTP compared to Control ewes, the effect sizes (Cohen's D) being of large and moderate magnitude respectively while not reaching significance (Figure 1). There was no difference in the measures of 11-OH-androstenedione, progesterone, testosterone, 17-OH-pregnenolone, 17-OH-allopregnanolone, androsterone, pregnenolone, allopregnanolone and androstenediol between BTP and Control ewes. The steroids 11-OH-testosterone, 11-ketoandrostenedione, 11-ketoprogesterone, 11-ketotestosterone, 11-OH-progesterone, 18-OH-corticosterone, 18-oxocortisol, 21-deoxycortisol, aldosterone, estriol, estradiol, estrone were not detected in any of the samples analyzed. A schematic representation of the steroidogenesis pathway, highlighting the steroid hormones affected by BTP exposure is illustrated in Supplementary Figure S1. The steroid differences evidenced in BTP ewes were not fetal sex-specific, with ewes carrying either sex showing similar concentrations (Supplementary Table S1).

3.2. Effect of biosolids exposure on maternal oxidative stress markers: Exposure to biosolids increased the mean plasma reactive oxygen metabolite concentration in BTP ewes compared to the Controls. In contrast, total antioxidant status, malondialdehyde, chlorotyrosine, nitrotyrosine and dityrosine

206 concentrations in plasma did not differ significantly in BTP ewes compared to Controls (Table 2). There
207 was no fetal sex-specific difference in the maternal plasma concentrations of any of the oxidative stress
208 markers (Supplementary Table S2).
209
210 3.3. Effect of biosolids exposure on maternal cytokines: The mean concentrations of the 14 cytokines in
211 the Control and BTP groups are listed in Table 3. BTP ewes had lower concentrations of the
212 proinflammatory cytokines IL-1β and IL-17A and the anti-inflammatory IL-36RA compared to the
213 Controls (Figure 2). The concentrations of IL-8 and MIP-1β were lower in BTP ewes, with the effects
214 sizes being of large and moderate magnitude difference, respectively; however, these did not reach
215 significance. Biosolids exposure had no impact on the concentrations of the inflammatory markers IL-
216 1α, IL-4, IL-6, IL-10, MIP-1α, IFN-γ, IP-10, TNF-α and VEGF-A. Ewes with female fetus had lower
217 concentrations of IP-10 than those carrying a male fetus in the BTP group (Supplementary Table S3).
218 There was no fetal sex-specific difference in the concentrations of any of the other cytokines
219 (Supplementary Table S3). VEGF-A showed significant interaction between biosolids exposure and
220 fetal sex, although its concentrations were not significantly different between the Control and BTP
221 groups or between ewes carrying a male or female fetus.
222
223 3.4. Effect of biosolids exposure on the correlation between maternal steroids, oxidative stress markers
224 and cytokines: The effect of biosolids exposure on the relationships between steroids, oxidative stress
225 markers and cytokines is represented in Figure 3. Several significant correlations were identified in the
226 Control ewes which were either lost or changed direction in the BTP ewes. Among the oxidative stress
227 markers, TAS and chlorotyrosine was negatively correlated with the corticosteroids in Control ewes but
228 these correlations were lost in the BTP ewes. TAS also showed a negative correlation with the cytokine

IL-6, while ROM was positively correlated with IL-4, IL-6 in Control ewes, but these correlations were disrupted by BTP exposure. Chlorotyrosine showed strong positive correlation with several cytokines exclusively in the BTP ewes. In the BTP group, ROM correlated with the pro-inflammatory cytokines IL-8 and MIP-1β. Among the Control animals, IL-36RA, VEGFA and IL-4 showed negative correlations with the steroid hormones. In contrast, IFN-γ, IL-1α, IL-4, IL-6, IL-10, IL-8, IL-36RA, TNF-α and VEGFA showed positive correlations with the steroid hormones in BTP ewes. The strong positive correlations of the sex steroids with the glucocorticoids and mineralocorticoids were also lost in the BTP ewes.

A difference in the pattern of correlation of cytokine with the steroids was evident based on the fetal sex and is represented in Figure 4. Animals with male fetus in the Control group showed a strong negative correlation between the steroid hormones and anti-inflammatory cytokine, IL-36RA; but this correlation was altered in the BTP exposed group. BTP exposure was associated with increased correlation between steroid hormones and several cytokines in animals with female fetus, that was absent among Control animals. The correlation between oxidative stress markers and steroid hormones was observed exclusively in BTP ewes carrying both male or female fetuses and was not sex specific.

4. Discussion:

This study indicates that combined preconceptional and gestational exposure to real-life low dose mixtures of ECs arising from grazing BTP causes changes in the maternal physiology, that might directly impact the intra-uterine environment, a key mediator of pregnancy outcomes, normal fetal development, and offspring health. This mid-gestational disruption in the maternal milieu is manifested at the level of steroidal, inflammatory and oxidative stress systems and their interrelationships. Fetal sex-specific impact on these maternal mediators was restricted to cytokine IP-10 but not the other

252 cytokines, steroids or oxidative stress markers. Previous studies have provided evidence that grazing on
253 BTP leads to perturbations in the maternal metabolome [34] and disruptions in offspring metabolic and
254 reproductive health manifested as differences in bone mineral density, and differences in the
255 development of the thyroid, reproductive neuroendocrine axis, testis and ovary [86-94]. The implication of
256 our present findings relative to the impact of biosolids exposure on the maternal milieu to the phenotypic
257 alterations reported previously in offspring of BTP-sheep is discussed below.
258
259 4.1. Preconceptional and gestational biosolids exposure disrupts maternal steroid milieu: Biosolids
260 exposure was associated with an increase in androgens (DHEA and androstenedione) and corticosteroids
261 (deoxycorticosterone, corticosterone, 11-deoxycortisol, cortisol and cortisone) in the maternal plasma.
262 Both androgens [95] and corticosteroids [96] are known developmental programming agents linked to
263 offspring metabolic and reproductive outcomes [97-99]. Maternal androgen excess, seen in the present
264 study, may be a contributor to disruptions in the maternal metabolic environment that we recently
265 reported in the same group of BTP animals [34]. Support for this premise comes from another sheep model
266 of gestational androgen excess, which also found disruptions in the maternal free fatty acids and acyl
267 carnitine profile [100]. Considerable evidence exists to support the involvement of excess gestational
268 androgens in reprogramming of the offspring reproductive neuroendocrine system [101-103], an aspect also
269 disrupted in the BTP model [87-89]. Similarly, the gestational increase in androgen evidenced in the current
270 study may have also contributed to perturbations in the testicular phenotype observed previously in BTP
271 exposed animals in separate cohorts/breeds [90,91,104] and in the testes of the offspring of ewes from the
272 same cohort as in the present study [91]. This effect is also supported by our studies of prenatal androgen
273 excess in sheep which demonstrated alterations to hypothalamic-pituitary-testicular axis dynamics [105-
274 107]. Given that our earlier studies in sheep found gestational androgen excess perturbs expression of

genes and proteins involved in fetal ovarian development [108-110] and follicular development [111], the increase in maternal androgen excess evidenced in the present study may have contributed to the ovarian disruptions seen in the offspring of BTP sheep [86,94,112]. The findings that maternal androgen excess impacts offspring ovarian function extends to other animal models of androgen excess as well [113,114]. In the present study, the androgens most influenced by EC exposure were DHEA and androstenedione; prenatal DHEA treatment has been shown to result in abnormal ovarian differentiation [115] in female rats. While high maternal concentrations of androstenedione is associated with masculinization in female guinea pigs [116], its effect on offspring phenotype is unknown. Although there is limited information on the specific programming effects of androstenedione and DHEA, both are precursors of testosterone and major source of androgen in peripheral tissues in women [117-119] and their increased levels in circulation is a manifestation of androgen excess[120]. The parallels drawn comparing findings from this study with other studies of androgen excess need to be considered taking into account differences in potencies.

Glucocorticoids, the corticosteroids produced by the adrenal cortex in response to stress, promote gluconeogenesis in liver and are critical for metabolic homeostasis and act as mediators of developmental programming [121,122]. The maternal increase in glucocorticoids evidenced in the present study may have also contributed to the altered testicular development and spermatogenic abnormalities seen in the offspring of BTP-sheep [90-92]. This possibility is supported by studies in sheep [123] and rats which indicate that gestational exposure to dexamethasone, a synthetic glucocorticoid that crosses the placenta to the fetus, perturbs testes development and spermatogenesis in offspring [124,125]. Similarly, the observed elevation of maternal glucocorticoids may have promoted the fetal ovarian perturbations previously reported in the offspring of BTP sheep [86,112] as gestational exposure to synthetic glucocorticoids (prednisone in mice and dexamethasone in rats) results in disrupted fetal ovarian morphology and function [126,127].

While data in the current study indicates effects of EC exposure on the steroidal milieu of the exposed mother, the importance of such gestational increases in androgens and glucocorticoids in altering development of the neuroendocrine-gonadal axis in offspring of BTP ewes remains to be determined. Evidence from human and animal studies also indicate that interactions between glucocorticoids and androgens may occur [128-130]. Pathological conditions of androgen excess are associated with an increase in circulating cortisol levels [131] while cortisol excess is associated with an increase in circulating androgen [132]. Lack of fetal-sex specific differences in maternal concentrations of any of the steroids reported in the present study is consistent with previous studies [133,134]. However, they contrast with a study which reported that women carrying a female fetus have increased maternal cortisol concentrations [68]. Overall, the steroid hormone changes in maternal circulation, in response to BTP exposure, seen in the current study may have resulted in elevated androgens and glucocorticoids in the intra-uterine environment. These alterations in the maternal steroid milieu may, therefore, serve as programming agents for the phenotypic abnormalities reported previously in offspring of BTP-exposed sheep.

4.2. Preconceptional and gestational biosolids exposure disrupts the maternal oxidative stress and inflammatory status: Biosolids exposure was associated with increased maternal oxidative stress as evidenced from the increase in concentrations of reactive oxygen species but not the antioxidant status in maternal circulation. Increased maternal oxidative stress may have contributed to the previously reported maternal metabolomic perturbations in BTP-sheep [34], as well as the phenotypic alterations in offspring of BTP sheep such as altered bone density [135], disruptions in the reproductive neuroendocrine axis [87-89], dysregulated gene and protein expression in liver [93], spermatogenic abnormalities [90], altered testis development [91,92,104], ovarian development [86,112] and pubertal timing (unpublished data). This is

supported by the observations that prenatal exposure to BPA increases maternal oxidative stress in sheep [64] and results in altered fetal ovarian development [136]. Likewise, prenatal exposure to PFAS that increases maternal oxidative stress [63] is also associated with perturbations in the maternal metabolome [137]. Phenotypic outcomes such as those reported in BTP offspring are also evidenced in pregnancies characterized by increased oxidative stress, like gestational diabetes [138-141] and preeclampsia [142]. Specifically, infants of mothers with gestational diabetes exhibit altered bone density [143] and pubertal timing [144], and altered offspring testes development [145]. Similarly, gestational diabetes [146] and preeclampsia [147] result in perturbations in the maternal metabolome which are similar to the metabolomic alterations seen in BTP-sheep. Additionally, maternal increase in oxidative stress in response to prenatal exposure to high-fat-diets is associated with ovarian disruptions in offspring [148]. In general, pathological pregnancies characterized by an increase in maternal oxidative stress are accompanied by a pro-inflammatory environment [54,149,150]. However, in BTP animals, the overall inflammatory status (i.e., whether it reflects pro-inflammatory or anti-inflammatory state) cannot be ascertained from the current data. A decrease in maternal concentrations of pro-inflammatory cytokines IL-1β, it's effector IL-17A, as well as a decrease in the anti-inflammatory cytokine IL-36RA were evident in BTP animals.

4.3. Preconceptional and gestational biosolids exposure perturbs the association between steroids, oxidative stress and inflammatory systems: Evidence points to a tight interrelationship between steroids and inflammation [151,152], oxidative stress and inflammation [38,149], and steroids and oxidative stress [153,154] in various physiological systems including pregnancy. Studies in humans and animals provide support of an interrelationship between sex steroids and glucocorticoids, an understudied mechanism in the context of developmental pathologies [128]. BTP exposure perturbed the positive correlation that existed between

the sex-steroids and glucocorticoids in the Control ewes, implicating a disruption in the interrelationship between them. Prior studies have shown that effect of exposure to ECs on immune cells is likely mediated via steroid-hormone dependent pathways [155], which could explain the perturbed correlation between steroids and cytokines in BTP-ewes. Individual concentrations of lipid peroxidation, nitrative stress and inflammatory markers did not vary between Control and BTP groups, but the directionality of their correlation with steroids was impacted by BTP-exposure in a fetal sex-specific manner reflective of the inter-relationship between the steroid, inflammatory and oxidative stress mediators in the maintenance of maternal homeostasis.

4.4. Strengths and limitations: There are several strengths to this study starting with the use of a large animal and translationally relevant exposure model to validate the effect of exposure to EC mixtures at environmentally relevant concentrations. This is also the first study to take a comprehensive look at perturbations in the maternal physiology, at multiple levels - steroid hormones, inflammatory and oxidative stress markers induced by pre-conceptional and gestational exposure to a real-life mixture of ECs present in biosolids. Most studies have explored the effects of gestational exposure to ECs on oxidative stress and inflammation primarily in the placenta and offspring [156-158], with limited studies exploring this relationship in maternal circulation [38,51,53,159]. To our knowledge, studies that have looked at all these three systems in response to chemical exposures are not available; ours is the first study in this regard. This is important because:1) all 3 are key contributors to maternal and offspring health, 2) they are all targets of EC impact and 3) there is potential for dialogue amongst them. Another strength is, sheep is an outbred, precocial animal model that is more translationally relevant compared to rodents [160],with a developmental trajectory similar to humans [161], thus offering a realistic assessment of risk from biosolids to human health.

Key limitations of this study include the lack of information on the exact EC composition of biosolids and the potential for differences in nutrient content of the grass based on the different fertilizer used[162]. These limitations have been addressed in detail earlier [34]. While the general composition [31] and the EC content of biosolids [163] used previously are available, the variability in EC composition amongst batches of biosolids and between locations would preclude which of the ECs contribute to the adverse effects seen with the use of biosolids. Lack of differences in the body condition scores of the control and BTP groups [34] indicates similar overall nutrition status between the treatment groups. As with outcomes in our earlier study using this model [92], there is inter-animal variability in the different maternal endpoints measured, probably because the susceptibility of animals to biosolids exposure varies. Nevertheless, this model exemplifies the real-life scenario as every human is exposed to a different composition of ECs at varying concentrations and they respond differently to these chemical exposures. Another limitation of this study is that the identified health impact of biosolids are merely associations that are suggestive in nature and do not represent causation or conclusive effects. Further studies using interventions like antioxidants are required to establish a causal relationship between the use of biosolids and observed perturbations. Although our analyses are consistent with a potential for inter-regulation amongst steroid, inflammatory and oxidative stress milieus, with changes in one milieu influencing another, the directionality of these interactions, which drives what, cannot be ascertained.

5. Conclusion:

Findings from this study indicate for the first time that pre-conceptional and gestational exposure to a 'real-life' EC mixture present in biosolids disrupts the maternal steroid, inflammatory and oxidative stress milieus, which may have affected the maternal physiology and contributes to programming of the phenotypic aberrations reported previously in the offspring of BTP sheep. Given the derivation and

composition of biosolids, these findings alert us to the human health risks of mixed EC exposure. The findings are likely to be of global significance as some of the changes seen in the maternal milieu are also shared with several human conditions including gestational diabetes and preeclampsia. While most available literature focuses on investigating these pathological pregnancies and EC exposure using a unidirectional approach, our results reiterate the importance of studying the role of steroidal, inflammatory and oxidative stress milieus in parallel, to delineate the maternal contributions to offspring phenotype.

6. References:

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Figure Captions:

Figure 1: Effect of BTP exposure on maternal steroid concentrations at mid gestation (Day 90).

Bar graphs represent mean values of steroids deoxycorticosterone, corticosterone, deoxycortisol, cortisone, cortisol, 16-OH-progesterone, androstenedione, dehydroepiandrosterone, 18-OH-cortisol and 17-OH-progesterone in Control (n=15) and BTP (n=15) ewes. Individual data points are represented

within the bar graph. ∗ denotes P <0.05 vs Control by unpaired t-test. ## denotes Cohen's d >0.8; # denotes Cohen's d >0.5.

Figure 2: Effect of BTP exposure on maternal plasma concentration of reactive oxygen metabolites and cytokines at mid gestation (Day 90). Bar graphs represent the mean values of reactive oxygen metabolites and the cytokines, IL-1β, IL-36RA, IL-17A, 1L-8, MIP-1β in Control (n=15) and BTP (n=15) ewes and IP-10 in Control and BTP ewes with male and female fetus. Individual data points are represented within the bar graph. ∗ denotes P <0.01 vs Control by unpaired t-test. ## denotes Cohen's d >0.8

Figure 3: Effect of biosolids exposure on the correlation between steroids, oxidative stress markers and cytokines measured in maternal plasma. Spearman correlation matrix between steroid levels, oxidative stress markers and cytokines at mid-gestation (Day 90) in Control and BTP groups. Pairwise correlations are depicted using the color spectrum with blue color indicating positive correlation and red color indicating negative correlation. Color intensity represents the strength of the correlation. Regions showing differences between Control and BTP groups are highlighted in the matrix. Only correlations with significance P < 0.05 and correlation coefficient r >0.5 or r< -0.5 are represented in the matrix.

Figure 4: Effect of biosolids exposure on the correlation between steroids, oxidative stress markers and cytokines in maternal plasma, based on fetal sex. Spearman correlation matrix between steroid levels, oxidative stress markers and cytokines at mid-gestation (Day 90) in (a) Control ewes with male fetus, (b) BTP ewes with male fetus, (c) Control ewes with female fetus and (d) BTP ewes with female fetus. The pairwise correlations are depicted using the color spectrum with blue color indicating positive

correlation and red color indicating negative correlation. Color intensity represents the strength of the correlation. Regions showing differences between Control and BTP groups are highlighted in the matrix. Only correlations with significance $P < 0.05$ and correlation coefficient $r > 0.5$ or $r < -0.5$ are represented in the matrix.

Table Captions:

Table 1: Effect of biosolids exposure on maternal plasma steroid levels at mid-gestation (Day 90).

Table 2: Effect of biosolids exposure on circulating oxidative stress marker levels at mid-gestation (Day 90).

Table 3: Effect of biosolids exposure on circulating cytokine levels at mid-gestation (Day 90).

Supplementary Figure S1: The schematic representation shows steroidogenesis pathways identifying steroid hormones affected by biosolids exposure. Steroid hormones that were increased by biosolids exposure are indicated in pink.

Supplementary Table S1: Descriptive statistics for steroid levels in Control and BTP maternal plasma comparisons based on fetal sex.

Supplementary Table S2: Descriptive statistics for oxidative stress markers in Control and BTP plasma comparisons based on fetal sex.

Supplementary Table S3: Descriptive statistics for cytokines in Control and BTP maternal plasma comparisons based on fetal sex.

Table 1: Effect of biosolids exposure on maternal plasma steroid levels at mid-gestation (Day 90).

Steroid	LLOQ (pg/mL)	Control (Mean ± SD)	BTP (Mean ± SD)	Fold change	P value	Cohen's d
Δ4 Steroids						
Deoxycortisol	2	145.7 ± 114.70	472.8 ± 419.40	3.24	**0.04**	1.06
Deoxycorticosterone	2	65.0 ± 20.26	96.4 ± 35.66	1.48	**0.01**	0.94
11-OH-Androstenedione	15	155.8 ± 53.15	167.2 ± 109.30	1.07	0.88	0.13
16-OH-Progesterone	20	90.2 ± 59.88	235.8 ± 168.10	2.62	**0.003**	1.15
17-OH-Progesterone	4	63.5 ± 31.38	88.1 ± 39.88	1.40	0.14	*0.70*
18-OH-Cortisol	2	1023.3 ± 585.64	1580.7 ± 717.67	1.60	0.05	0.91
Androstenedione	2	15.7 ± 4.71	17.9 ± 3.71	1.13	**0.04**	0.47
Corticosterone	3	220.4 ± 138.5	516.5 ± 331.60	2.35	**0.006**	1.17
Cortisol	7	17384.7 ± 12177.00	36848.7 ± 21841.6	2.18	**0.005**	1.13
Cortisone	3	2562.7 ± 1084.42	4080.0 ± 1439.15	1.58	**0.002**	1.20
Progesterone	2	6673.3 ± 2234.44	6595.3 ± 1607.05	0.99	0.92	0.05
Testosterone	1	36.0 ± 13.90	31.9 ±10.25	0.89	0.45	0.34
Δ5 Steroids						
17-OH-Pregnenolone	15	803.8 ± 756.37	1058.2 ± 892.24	1.38	0.1	0.36
17-OH-Allopregnanolone	8	9783.2 ± 10816.10	10389.3 ± 10020.60	1.02	0.35	0.02
Dehydroepiandrosterone	5	91.9 ± 125.71	1678.2 ± 1748.28	18.48	**0.03**	1.33
Androsterone	32	193.8 ± 213.24	267.7 ± 362.08	1.38	0.88	0.25
Pregnenolone	15	7515.0 ± 3788.53	8412.6 ± 4055.61	1.12	0.48	0.23
Allopregnanolone	4	563.3 ± 208.63	682.5 ± 193.40	1.21	0.25	*0.59*
Androstenediol	55	3772.1 ± 3132.15	5160.6 ± 3362.01	1.37	0.13	0.43

Significant differences are in bold. Cohen's d >0.8 (large magnitude difference) is highlighted in grey and Cohen's d >0.5-0.8 (medium magnitude difference) is shown in italics

Table 2: Effect of biosolids exposure on circulating oxidative stress marker levels at mid-gestation (Day 90).

Oxidative Stress Marker	Control (Mean ± SD)	BTP (Mean ± SD)	P value	Cohen's d
Reactive Oxygen Metabolites (μmol/L)	3.97 ± 1.360	5.66 ± 1.706	**0.006**	1.09
Total Antioxidant status (mmol/L)	1.04 ± 0.133	1.05 ± 0.103	0.81	0.08
Malondialdehyde (μM)	1.51 ± 1.079	1.80 ± 1.063	0.46	0.27
Chlorotyrosine (μM/mol of Y)	91.41 ± 33.86	83.90 ± 15.423	0.72	0.27
Nitrotyrosine (μM/mol of Y)	210.77 ± 99.672	218.83 ± 88.865	0.92	0.09
Dityrosine (μM/mol of Y)	36.96 ± 7.964	33.31 ± 5.762	0.11	*0.57*

Significant differences are in bold. Cohen's d >0.8 (large magnitude difference) is highlighted in grey and Cohen's d >0.5-0.8 (medium magnitude difference) is shown in italics.

Table 3: Effect of biosolids exposure on circulating cytokine levels at mid-gestation (Day 90).

Cytokine	LLOQ (pg/mL)	Control (Mean ± SD pg/mL)	BTP (Mean ± SD pg/mL)	Fold change	P value	Cohen's d
IL-1α	0.1	8.87 ± 7.487	7.66 ± 7.172	1.16	0.53	0.16
IL-1β	6.8	18.57 ± 15.105	7.25 ± 5.582	2.60	**0.02**	1.03
IL-4	4.9	9.21 ± 7.188	6.22 ± 4.780	1.48	0.14	0.49
IL-6	1.9	29.43 ± 12.180	27.23 ± 28.00	1.07	0.42	0.09
IL-8	3.5	9601 ± 10688	2818± 880.40	3.41	0.86	6.90
IL-10	2.2	123.30 ± 60.530	134.30 ± 99.100	0.92	0.99	0.13
IL-17A	2.8	2.95 ± 2.043	1.70 ± 1.518	1.76	**0.01**	*0.74*
IL-36RA	1.0	89.27 ± 29.980	57.84 ± 29.560	1.53	**0.02**	1.03
IFN-γ	1	1.55 ± 0.956	1.26 ± 0.593	1.23	0.41	0.38
IP-10	1.6	945.80 ± 324.400	892.30 ± 252.200	1.06	0.78	0.19
MIP-1α	33.8	580.20 ± 358.600	669.60 ± 541.600	0.87	0.99	0.20
MIP-1β	3.4	2.99 ± 1.465	2.38 ± 0.083	1.25	0.41	*0.57*
TNF-α	12.5	314.90 ± 224.20	265.4 ± 210.80	1.19	0.48	0.23
VEGF-A	1.26	73.04 ± 33.410	64.88 ± 35.89	1.12	0.27	0.23

Significant differences are in bold. Cohen's d >0.8 (large magnitude difference) is highlighted in grey and Cohen's d >0.5-0.8 (medium magnitude difference) is shown in italics.

Figure1

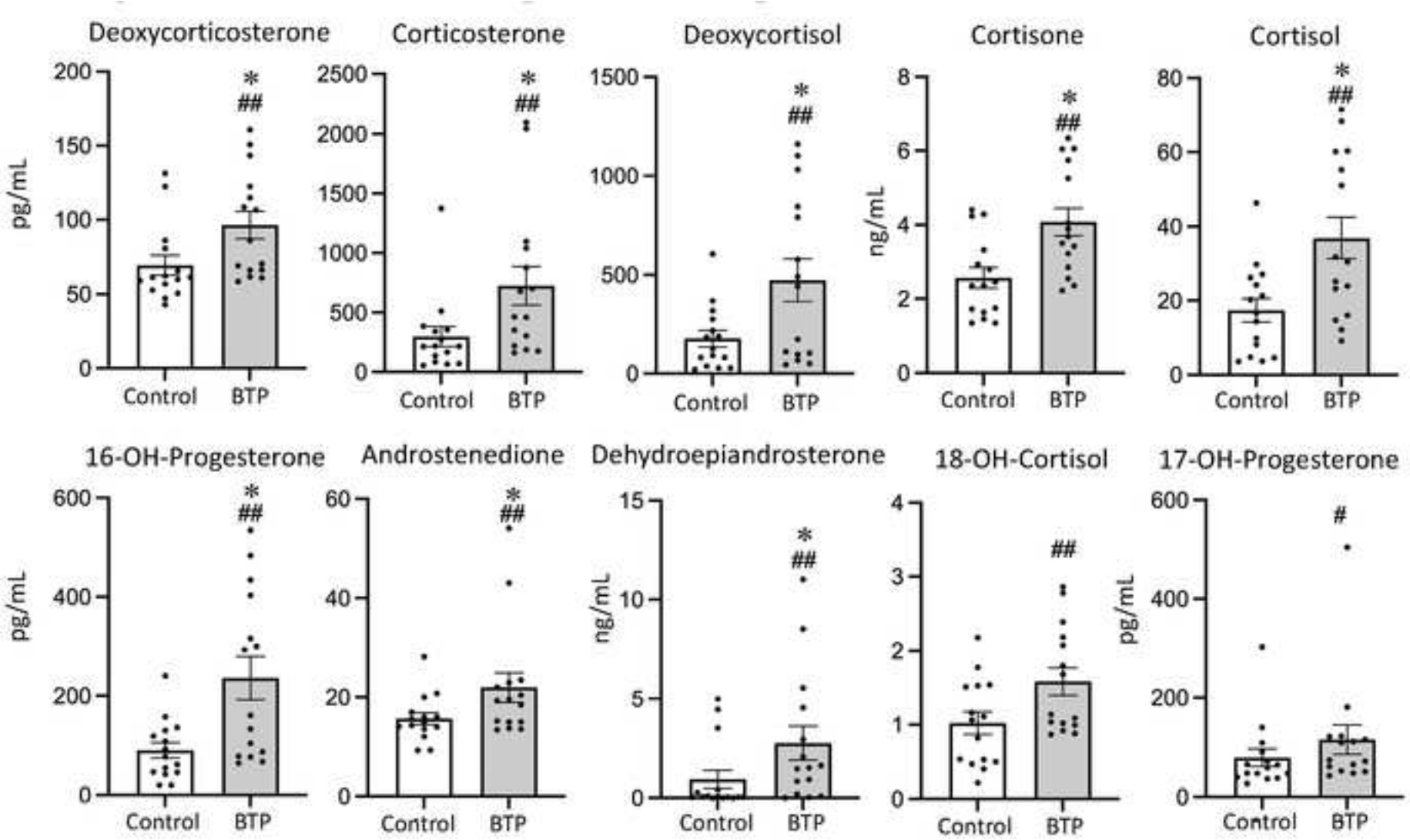


Figure2

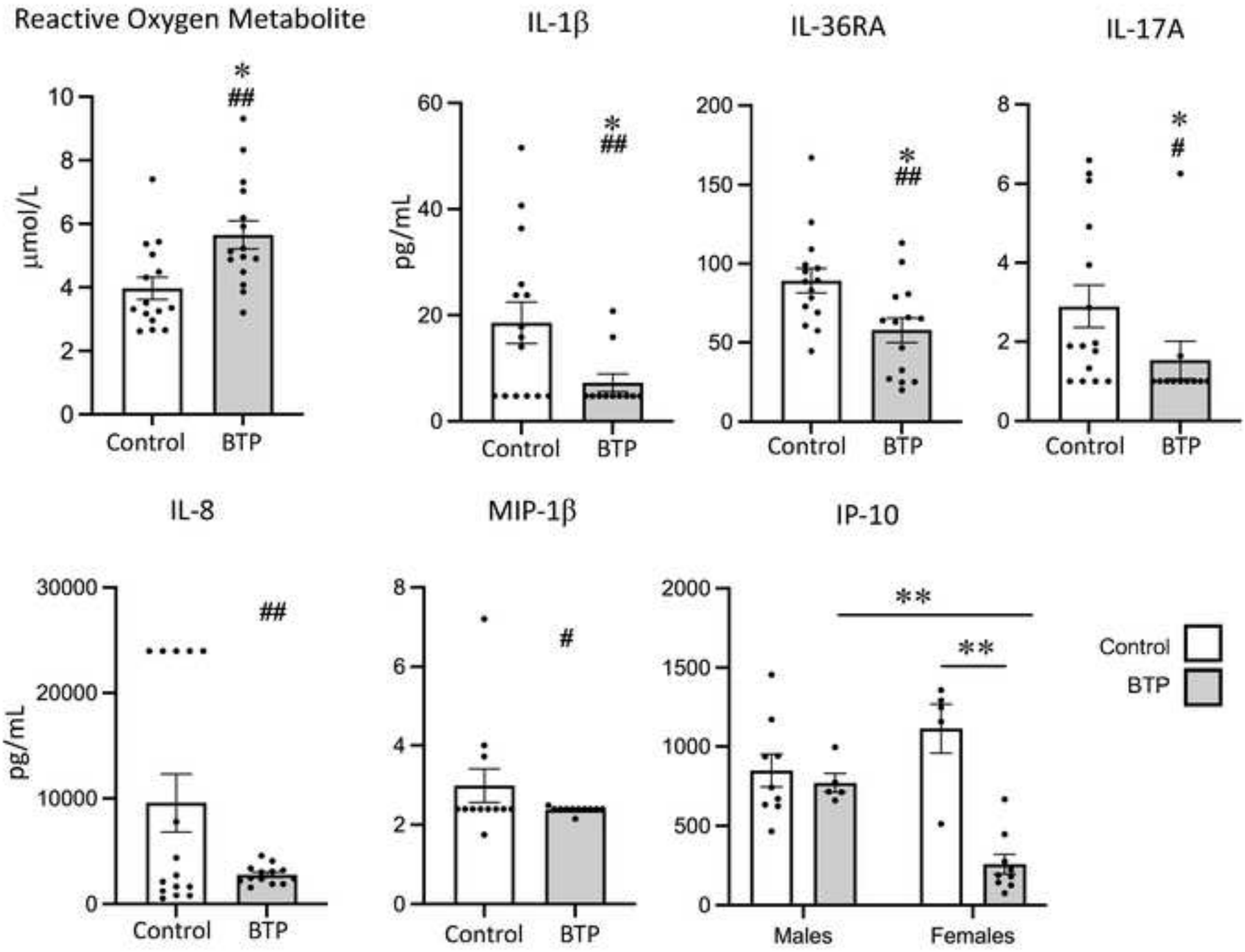


Figure3

Click here to access/download;Figure;Figure3_pdf format.pdf

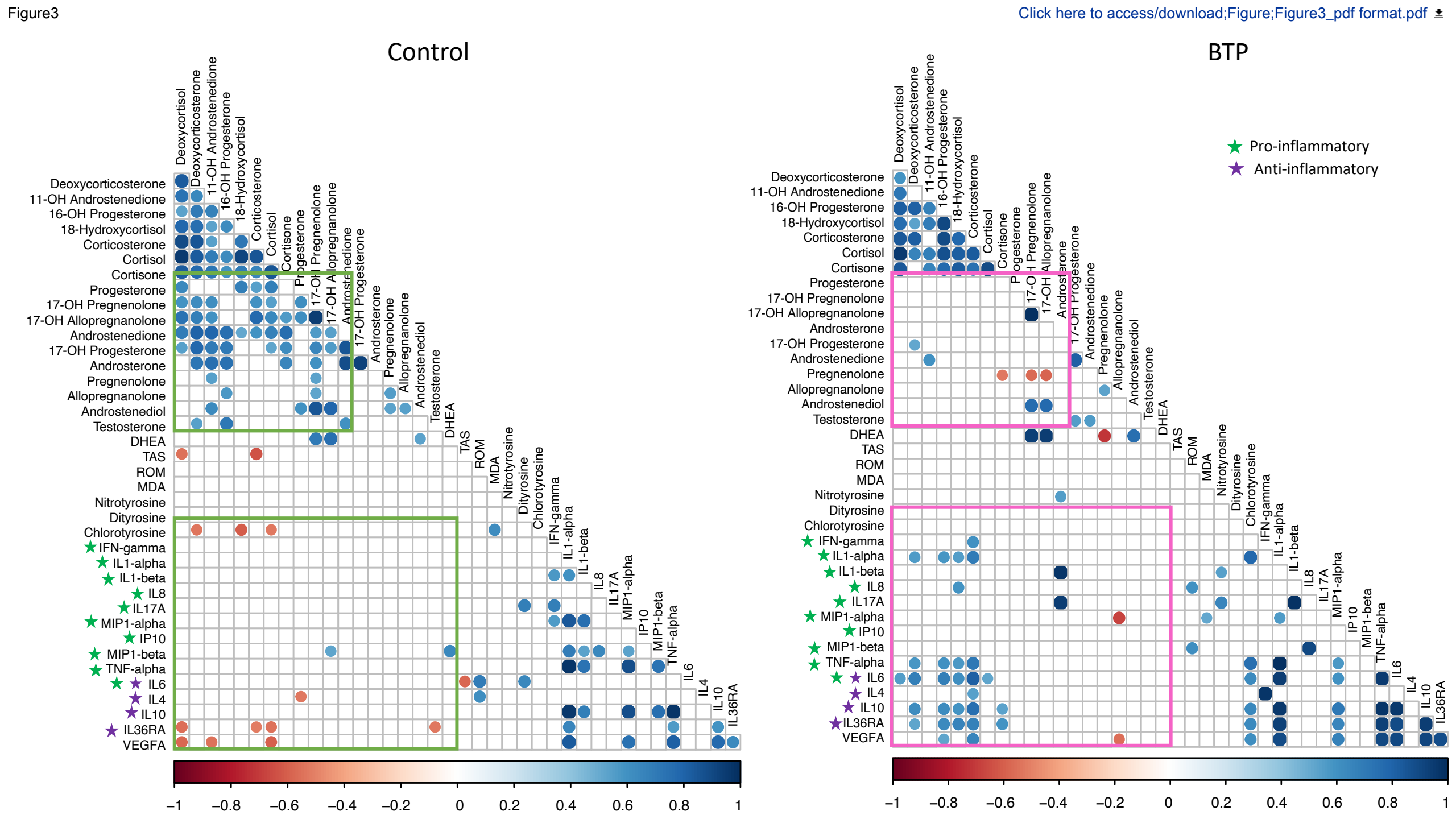
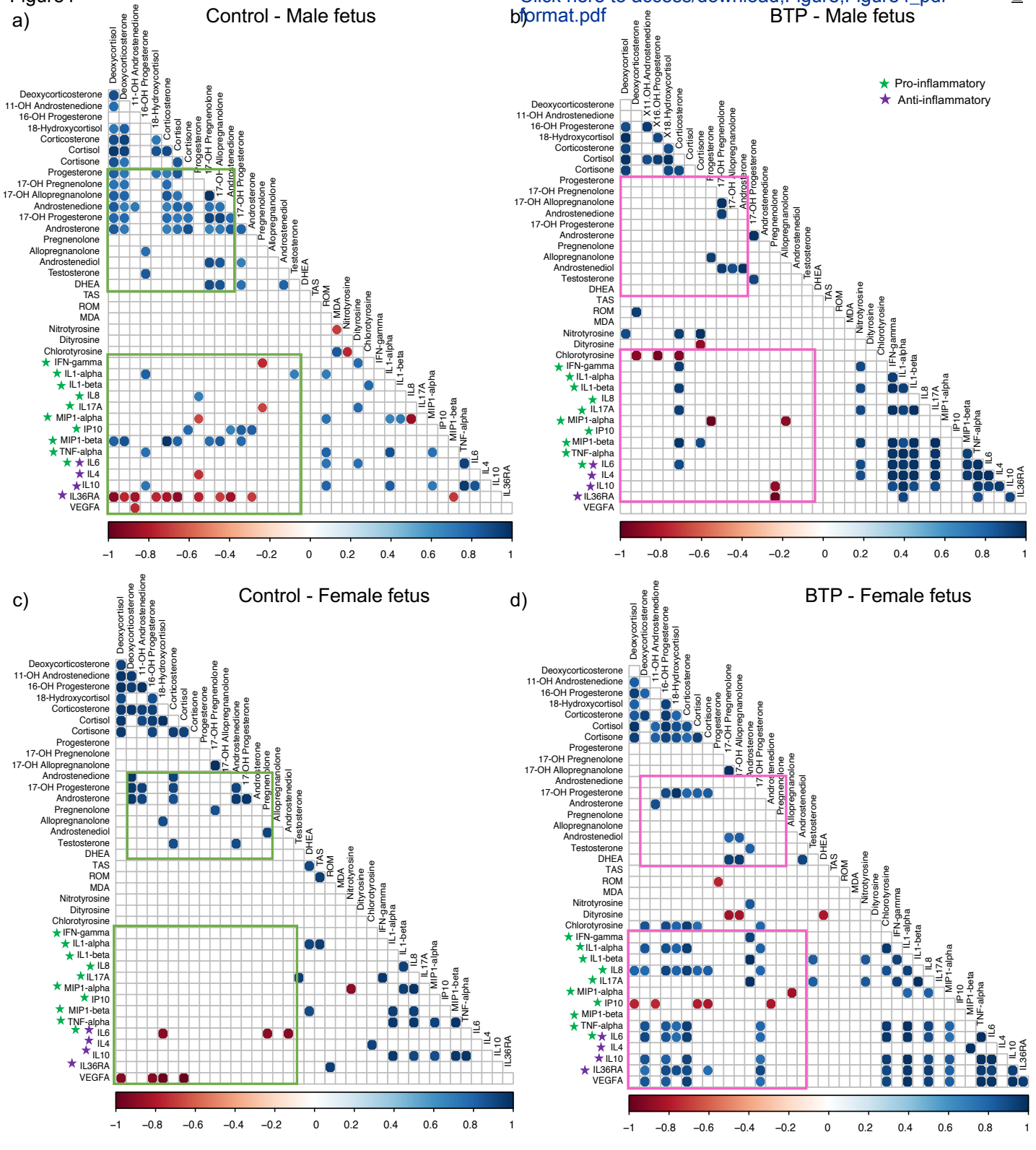


Figure4



Supplementary Figure S1

Declaration of interests

☒The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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Soundara Viveka Thangaraj: Methodology, Analysis, Visualization, Writing- Original draft preparation.
Lixia Zeng: Investigation
Subramaniam Pennathur: Investigation, Writing - Review & Editing
Richard Lea: Writing - Review & Editing
Kevin Sinclair: Funding, Writing - Review & Editing
Michelle Bellingham: Investigation, Writing - Review & Editing
Neil Evans: Funding, Investigation, Writing - Review & Editing
Auchus Richard: Investigation, Writing - Review & Editing
Vasantha Padmanabhan: Funding, Conceptualization, Methodology, Supervision, Writing – Original Draft, Review & Editing