

Estradiol and dihydrotestosterone exert sex-specific effects on human fibroblast and endothelial proliferation, bioenergetics, and vasculogenesis

Corresponding Author: Dr Mark Mondrinos

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The authors present exciting and important findings demonstrating how E2 and DHT levels in culture media influence male and female fibroblast and endothelial cell proliferation, mitochondrial function, and tissue vasculogenesis. I have a few concerns and suggestions that I believe would strengthen the manuscript's impact:

1) The use of multiple doses of E2 and DHT to assess dose-response effects is a strong aspect of the study. However, I think the conclusions would be further supported by including a comparison not only to phenol red-free, charcoal-stripped FBS without added hormones, but also to standard culture conditions (i.e., with phenol red and regular FBS). This additional condition would better contextualize your findings within common laboratory practices and underscore the importance of adopting sex-specific culture conditions.

2) I appreciated the discussion point noting that E2 and DHT are not exclusive to females and males, respectively, and that future work will address this complexity. Including a brief discussion of how the doses used relate to physiological hormone levels in both sexes would add valuable context. For example, this could clarify whether your E2 concentrations model premenopausal and/or postmenopausal conditions.

3) In Figure 2D, the HRMVEC images for XX versus XY vehicle controls appear morphologically distinct, with XX cells looking less "endothelial." Is this expected? It raises the question of whether XX HRMVECs may have started undergoing an endothelial-to-mesenchymal transition (EndMT), which could influence proliferation and mask hormonal effects. You may want to consider using different representative images, or considering EndMT as a possibility if all XX HRMVECs share that more elongated morphology.

4) For the bar and box plots, I recommend standardizing the color schemes across figures for each condition to aid visual clarity. Additionally, Figures 6 and 7 might be better combined, or at least presented with consistent y-axes. This would make it easier for readers to compare vasculogenesis between male and female conditions directly.

Reviewer #2

(Remarks to the Author)

The article by Martier et al. investigated the action of estradiol and testosterone on human primary cells. The study assessed effects on two cell types (fibroblasts and endothelial cells) from several tissues (lung, ocular, umbilical, and microvascular) from four donors of each sex per cell type. Dose-response behavior of each cell type and sex for both sex hormones was assessed by measuring cell proliferation, expression of select genes associated with energetics and stress-response, mitochondrial membrane potential, and in vitro vasculogenesis. The article expands the field's understanding of sex hormone effects on relevant cell types in culture, demonstrating the need for accounting for sex in cell culture systems and sex-specific media formulations. The efforts by the authors are recognized and commended for compiling the extensive dataset. That said, there are some areas for improvement and of concern that require clarification and expansion.

1. Introduction. Missing from the introduction is mention of the time-dependent (age-dependent) nature of sex hormones as well as mention of the basal/serum concentrations of sex hormones in males and females. This information is necessary for understanding and contextualizing the need for recognizing sex hormones in cell culture systems and the range of concentrations of E2 and DHT investigated.
2. Methods. 2D Cell Culture. Were the cells from the different donors pooled or used individually. This should be specified. Additionally, the lot numbers and characteristics of each donor cell population should be included in the SI. On this point, Supplemental Figure 3 shows the SRY gene expression for confirming cell sex. It is concerning that four XX cell populations had SRY expression in fact greater than any XY cell population. Were these XX cell populations with SRY expression used in the study? If so, their data should be removed. Inappropriately sexed cells from commercial vendors happens and it was good practice by the authors to confirm whether the reporting from the vendor was accurate. As such, any data generated from these four XX populations with SRY expression should not be included in the study.
3. Methods. Ki67 immunofluorescence. Additional details are needed. Please clarify the vendor and type of media and serum mentioned, "phenol red-free media supplemented with 2% charcoal stripped serum...", include lot numbers for the serum, as well. This comments applies elsewhere, too. Please specify why the exposure was for 48 hrs? Define PBS. Specify the lot number for the "rabbit anti-Ki67 antibody". Specify the fluorescent tag on the phalloidins. Specify the grade of ethanol (e.g., molecular biology, GC, etc.). Specify whether vehicle controls were included.
4. Methods. qRT-PCR. Please use consistent nomenclature throughout either qRT-PCR or RT-qPCR. Specify the grade of ethanol (e.g., molecular biology, GC, etc.). Was the media and serum the same as the other assays?
5. Methods. Tetramethylrhodamine mitochondrial membrane potential assay. Define TMRM. Why was replication only specified for this assay and not the others?
6. Methods. Fabrication of PDMS microarrays. Please specify details on the polyurethane coating of the molds. For the sentence, "The ideal amount of PDMS for our purpose was found to be 0.25% by weight as this endowed the material...", specify whether the "PDMS" mentioned is the PDMS modified with PEG. The sentence, "To ensure complete attachment of the collagen gels to the PDMS devices...", requires additional context. As written it does not logically follow.
7. Methods. 3D vasculogenesis assay. Define PDA. Provide reasoning for the hydrogel composition. Please correct "nm" to "nM" and state the solvent used for DHT.
8. Methods. Immunofluorescence and 3D whole mount staining. DAPI was already defined previously and the fluorophore tag on the phalloidins should be specified.
9. Methods. Imaging and image analysis. Three images per sample seems a bit low. How was the location in the sample selected for imaging? Additionally, the details on image number appear inconsistent: "For 2D cultures, 3 images of each sample were taken to create 3 technical replicates," and "This process was completed for each of the 5 images taken from each well. The average of these 5 measurements was then used as the average intensity across the entire well." Please specify the dataset that the neural network was pretrained on.
10. Methods. Statistical analysis. ANOVA is not the best statistical method for analyzing a mixed model of categorical (sex) and continuous variable (concentration). A regression should be used instead. Additionally, a statement on the extent to which the data satisfied the assumptions required for ANOVA/regression should be included.
11. Results. Sex-specific hormone effects on fibroblast and endothelial cell proliferation. More details should be included as to why proliferation was the target end point measure for the action of sex hormones on these cell types. Additionally, additional details should be included as to why the four cell types were investigated. How do the concentrations of sex hormones dosed compare to those found endogenously. How was the "Ki67 index" calculated, what does it mean? It appears to simply be the percentage of Ki67 positive cells in which case it should just be called as such.
12. Results. Figures 1,2, 4, 5, 6, 7, 8. The bar graphs should be plotted as X-Y plots because dose is a continuous variable. Additionally can the data be disaggregated and analyzed by donor cell population (see <https://advanced.onlinelibrary.wiley.com/doi/full/10.1002/adhm.202400039>).
13. Results. Figure 3. Is there a positive control that could be used to compare these expression levels. Additionally, what is the variance of the expression levels because the effects appear to be at most 2 fold. Were differences in expression statistically significant? The figure is also quite small consider separating it out so that it is more legible.
14. Results. Sex-specific effects of E2 and DHT on bulk tissue vasculogenesis. Regarding the PEG-PDMS absorption of the sex hormones (Figure S1), there is still Nile red staining with the PEG-PDMS polymer. Can the amount of absorbed Nile red be quantified so that an effective dose be determined for the sex hormones. How comparable is Nile red partitioning to that of the sex hormones (e.g., values of K_{ow}).
15. Discussion. Broadly the discussion should include greater reference and contextualization with the growing literature of sex-specific cell studies for which there is an abundance for endothelial cells particularly HUVECs including,
 * <https://doi.org/10.1161/01.CIR.91.3.75>
 * [10.1007/s40618-014-0118-1](https://doi.org/10.1007/s40618-014-0118-1)
 * <https://doi.org/10.1038/s41598-017-10139-x>
 * <https://doi.org/10.1007/s10456-016-9525-6>
 * <https://doi.org/10.1113/JP276048>
 * [10.3390/cells10102564](https://doi.org/10.3390/cells10102564)
 * <https://doi.org/10.1016/j.mbsplus.2024.100145>
 * <https://doi.org/10.1093/cvr/cvy185>

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The authors have addressed all of my major comments and concerns from the first version I reviewed. I think the updates, especially the changes to the figures, make for a much stronger manuscript. I do not have any additional edits to suggest.

Reviewer #2

(Remarks to the Author)

The authors have adequately addressed my comments and concerns. This work is a valuable addition to the field.

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Reviewers' comments

Reviewer #1 (Remarks to the Author):

The authors present exciting and important findings demonstrating how E2 and DHT levels in culture media influence male and female fibroblast and endothelial cell proliferation, mitochondrial function, and tissue vasculogenesis. I have a few concerns and suggestions that I believe would strengthen the manuscript's impact:

Thank you for the supportive feedback and helpful comments. We believe that addressing the thoughtful points you raised significantly improved the clarity and impact of our paper.

1) The use of multiple doses of E2 and DHT to assess dose-response effects is a strong aspect of the study. However, I think the conclusions would be further supported by including a comparison not only to phenol red-free, charcoal-stripped FBS without added hormones, but also to standard culture conditions (i.e., with phenol red and regular FBS). This additional condition would better contextualize your findings within common laboratory practices and underscore the importance of adopting sex-specific culture conditions.

Thank you for this helpful comment. We noted that 2D assay readouts at 48-hour and 7-day time points, i.e. PCR assays and simple stains, showed no significant difference in any metrics measured between 1nM E2 and the FBS groups. As such, we omitted this data from other analyses, but we have now included the PCR data comparing the FBS and 1nM E2 groups in new Supplemental Figures 3 and 4. More generally, we appreciate the insight that comparison to standard FBS containing media is imperative. To that end, we have an additional manuscript under revision for publication in a different journal that focuses on comparing sex differences in the response to FBS and CSS (without exogenous hormones) for a wide range of metrics.

2) I appreciated the discussion point noting that E2 and DHT are not exclusive to females and males, respectively, and that future work will address this complexity. Including a brief discussion of how the doses used relate to physiological hormone levels in both sexes would add valuable context. For example, this could clarify whether your E2 concentrations model premenopausal and/or postmenopausal conditions.

Thank you for pointing out this important detail. We have included additional discussion of known physiological hormone levels and the corresponding rationale for selection of the dose ranges utilized in our studies. We also included discussion of the challenges associated with using serum levels as a framework for determining media concentrations for modeling a desired physiological state. While serum levels may be appropriate for blood endothelial cells, cells like fibroblasts and parenchymal cells in a given tissue are seeing the concentration in the interstitial fluid – and these concentrations are not known. It is an open question, but we believe that our 1 nM E2 condition is reflective of premenopausal (~270 pg/ml) and 0.1 nM (~27 pg/ml) is still above the < 10-20 pg/ml cutoff for postmenopausal. Please see lines 68-76 in the Introduction.

3) In Figure 2D, the HRMVEC images for XX versus XY vehicle controls appear morphologically distinct, with XX cells looking less “endothelial.” Is this expected? It raises the question of whether XX HRMVECs may have started undergoing an endothelial-to-mesenchymal transition (EndMT), which could influence proliferation and mask hormonal effects. You may want to consider using different representative images, or considering EndMT as a possibility if all XX HRMVECs share that more elongated morphology.

We have consistently noted that HRMVEC (and other microvascular endothelial cells not used in this study) exhibit a more elongated/spindly morphology in comparison to endothelial cells collected from larger vessels, such as HUVEC. This is in line with others who have reported a

more elongated morphology in microvascular endothelial cells, as well as significant differences in morphology between donors with no significant impacts on functional outputs (PMID: 39996794, 8412854). Regarding the morphological difference between XX and XY HRMVEC, in our hands, XX microvascular cells exhibit a slightly more exaggerated spindle shape relative to their XY counterparts, but they continue to support vasculogenesis and endothelial barrier formation – typically to an enhanced degree relative to XY counterparts. The other manuscript mentioned in response to comment #1 above highlights that FBS potently activates XX endothelial cells relative to XY cells, and we believe that the expansion culture period in FBS-containing medium, and the transition period after plating in FBS before switching to CSS + hormones in our experiments may be the cause of the altered morphology (more activated) in XX endothelial cells. We are currently working on culture methods that eliminate the use of FBS at any stage by supplementing CSS with a wider range of exogenous factors beyond the sex hormones that enable successful expansion culture and plating, while also hopefully eliminating the differences between XX and XY cell performance that we believe are due to the presence of FBS at various stages of culture and experimentation. All of that said, the original image panels showed an exaggerated example of this morphological difference. We included new images for these panels that are closer to confluence and the center of the well to showcase a more representative morphology. We have also written additional language to clarify morphological heterogeneity in cultured endothelial cells in the Results section (please see lines 330-334).

4) For the bar and box plots, I recommend standardizing the color schemes across figures for each condition to aid visual clarity. Additionally, Figures 6 and 7 might be better combined, or at least presented with consistent y-axes. This would make it easier for readers to compare vasculogenesis between male and female conditions directly.

We agree that inconsistent color schemes hampered clarity, making it more difficult to interpret and compare data across Figures. We have accordingly updated all Figures, using a standard color scheme throughout. We appreciate the recommendations on improving Figures 6 and 7. We opted to maintain 6 and 7 as independent figures. We standardized the axes and increased the size of the plots to better enable direct comparison of XX and XY vasculogenesis.

Reviewer #2 (Remarks to the Author):

The article by Martier et al. investigated the action of estradiol and testosterone on human primary cells. The study assessed effects on two cell types (fibroblasts and endothelial cells) from several tissues (lung, ocular, umbilical, and microvascular) from four donors of each sex per cell type. Dose-response behavior of each cell type and sex for both sex hormones was assessed by measuring cell proliferation, expression of select genes associated with energetics and stress-response, mitochondrial membrane potential, and in vitro vasculogenesis. The article expands the field's understanding of sex hormone effects on relevant cell types in culture, demonstrating the need for accounting for sex in cell culture systems and sex-specific media formulations. The efforts by the authors are recognized and commended for compiling the extensive dataset. That said, there are some areas for improvement and of concern that require clarification and expansion.

We greatly appreciate your thorough review of our manuscript and the detailed comments. We believe that your comments and suggestions helped us significantly improve the clarity and overall impact of our paper.

1. Introduction. Missing from the introduction is mention of the time-dependent (age-dependent) nature of sex hormones as well as mention of the basal/serum concentrations of sex hormones in males and females. This information is necessary for understanding and contextualizing the

need for recognizing sex hormones in cell culture systems and the range of concentrations of E2 and DHT investigated.

Thank you for pointing out the need to better clarify the scientific rationale for our general approach and the specific ranges of hormone concentrations investigated in our study.

Language about the average levels of hormones in males and females as well as discussion about the changes in hormone levels throughout the lifespan in both sexes has been added to the Introduction (see also response to Reviewer 1's comment #2). Please see lines 68-76 in the Introduction.

2. Methods. 2D Cell Culture. Were the cells from the different donors pooled or used individually. This should be specified. Additionally, the lot numbers and characteristics of each donor cell population should be included in the SI. On this point, Supplemental Figure 3 shows the SRY gene expression for confirming cell sex. It is concerning that four XX cell populations had SRY expression in fact greater than any XY cell population. Were these XX cell populations with SRY expression used in the study? If so, their data should be removed. Inappropriately sexed cells from commercial vendors happens and it was good practice by the authors to confirm whether the reporting from the vendor was accurate. As such, any data generated from these four XX populations with SRY expression should not be included in the study.

We have now included a general "Experimental Design" section to the beginning of methods which states clearly that cells were used individually and not pooled. A new Supplemental Figure has been added that shows lot numbers and donor information for each cell lot utilized. In response to the data shown in the original Supplemental Figure 3 (now Supplemental Figure 2), the SRY gene expression is shown at cycle threshold values. Per our PCR system, a cycle threshold number greater than 35 equates to background expression and is not to be taken as a true positive. Accordingly, the 4 XX populations that did express any SRY positivity were well over this cutoff and can therefore be considered negative for SRY expression. This supports that these cells were correctly sexed as XX by the suppliers and were therefore included in the studies.

3. Methods. Ki67 immunofluorescence. Additional details are needed. Please clarify the vendor and type of media and serum mentioned, "phenol red-free media supplemented with 2% charcoal stripped serum..." include lot numbers for the serum, as well. This comments applies elsewhere, too. Please specify why the exposure was for 48 hrs? Define PBS. Specify the lot number for the "rabbit anti-Ki67 antibody". Specify the fluorescent tag on the phalloidins. Specify the grade of ethanol (e.g., molecular biology, GC, etc.). Specify whether vehicle controls were included.

Language clarifying the media and the serum utilized as well as the ethanol used in hormone studies has been added to the new "Experimental Design" section of methods.

Additional clarifications have been made, including definition of PBS, lot number of Ki67, fluorescent tags on phalloidins, ethanol grade, and vehicle control statements among other additional information regarding suppliers and catalog numbers throughout. We selected 48 hours as our endpoint for Ki67 and other 2D assays based on RT-qPCR results showing there was a significant effect of exogenous hormones within 48 hours. We believe that the 48-hour time point also helps limit the influence of trophic effects from altered phenotypes induced by hormone stimulation in more extended cultures. Language clarifying these points have been added to the manuscript (please see lines 124-127).

4. Methods. qRT-PCR. Please use consistent nomenclature throughout either qRT-PCR or RT-qPCR. Specify the grade of ethanol (e.g., molecular biology, GC, etc.). Was the media and serum the same as the other assays?

We apologize for the inconsistent nomenclature. Terminology and acronyms are now consistent throughout the manuscript. See the new “Experimental Design” section of the Methods for expansion of details regarding media and ethanol utilized.

5. Methods. Tetramethylrhodamine mitochondrial membrane potential assay. Define TMRM. Why was replication only specified for this assay and not the others?

The TMRM acronym is now defined clearly in the corresponding Methods section. All replication data for each assay has now been included in the corresponding Methods sections and Figure Legends.

6. Methods. Fabrication of PDMS microarrays. Please specify details on the polyurethane coating of the molds. For the sentence, “The ideal amount of PDMS for our purpose was found to be 0.25% by weight as this endowed the material...,” specify whether the “PDMS” mentioned is the PDMS modified with PEG. The sentence, “To ensure complete attachment of the collagen gels to the PDMS devices...,” requires additional context. As written it does not logically follow. We previously reported methods for polyurethane clear coating of 3D printed SLA resin molds used for PDMS soft lithography. We have now clearly highlighted the relevant citation to direct readers to those detailed manufacturing methods. Additional context for PDA treatment has been added for clarification. Please see lines 193-196 in Methods.

7. Methods. 3D vasculogenesis assay. Define PDA. Provide reasoning for the hydrogel composition. Please correct “nm” to “nM” and state the solvent used for DHT.

Thank you for pointing out these errors and omissions. PDA is now clearly defined, “nm” has been corrected to “nM”, and the solvent used for DHT is clearly stated. Reasoning for the composition of hydrogel has been added. Please see lines 216-217 in Methods.

8. Methods. Immunofluorescence and 3D whole mount staining. DAPI was already defined previously and the fluorophore tag on the phalloidins should be specified.

Definition of the fluorophore has been added.

9. Methods. Imaging and image analysis. Three images per sample seems a bit low. How was the location in the sample selected for imaging? Additionally, the details on image number appear inconsistent: “For 2D cultures, 3 images of each sample were taken to create 3 technical replicates,” and “This process was completed for each of the 5 images taken from each well. The average of these 5 measurements was then used as the average intensity across the entire well.” Please specify the dataset that the neural network was pretrained on.

We apologize for this error. For all imaging assays, 3 images per well were selected at randomized points avoiding the area adjacent to the walls of the well. We clarified description of captured image numbers in the corresponding Methods sections and Figure Captions for each imaging assay. Pretraining of the neural network used for vascular morphometry has been described in detail elsewhere, and appropriate citations to these publications have been added (line 259 in Methods).

10. Methods. Statistical analysis. ANOVA is not the best statistical method for analyzing a mixed model of categorical (sex) and continuous variable (concentration). A regression should be used instead. Additionally, a statement on the extent to which the data satisfied the assumptions required for ANOVA/regression should be included.

We believe that two-way ANOVA is an appropriate statistical test for this application. While concentration may be a continuous variable, we have selected to only analyze a set number of discrete concentration values; thus, it is appropriate to consider concentration as a categorical variable in our analysis. Additionally, we did not intend to ascertain a dose-response curve.

Instead, we are analyzing sex differences in the response to each concentration as a discrete treatment. We do not have adequate data to generate an entire dose response curve, nor do we have reason to assume a linear relationship between variables. Thus, we believe that regression analysis is not appropriate for this dataset. This statistical approach is supported by others in the literature (PMID: 17618310, 31145670). Additionally, our data met the necessary assumptions for two-way ANOVA (namely normality, homogeneous variance, independence of observations – each endpoint is discrete, no continuous time points for a given sample). Language specifying conformance with assumptions for two-way ANOVA have been added to the “Statistics and Reproducibility” Methods section (please see lines 271-274).

11. Results. Sex-specific hormone effects on fibroblast and endothelial cell proliferation. More details should be included as to why proliferation was the target end point measure for the action of sex hormones on these cell types.

Thank you for this helpful comment to clarify the rationale for our assays, cell type selection and culture conditions. Cell proliferation is central to many normal physiological processes (i.e., healing, regeneration, etc.) and disease pathogenesis (cancer, fibroproliferation, etc.). Driving cell proliferation is also necessary for most cell culture applications in biotechnology. Most notably, the significant impact of sex hormones on proliferation in many of these contexts has been established. We added language to explain this reasoning (please see lines 295-298).

Additionally, additional details should be included as to why the four cell types were investigated.

We focused on endothelial cells and fibroblasts as key cell types utilized in MPS models of the vasculature. We chose to look at multiple tissue sources to explore potential tissue-specific effects vs. more conserved responses. We added new language explaining this rationale for cell type selection in these studies. Please see lines 284-290 in Results.

How do the concentrations of sex hormones dosed compare to those found endogenously.

Language regarding the endogenous hormone ranges for E2 and DHT has been added to the Introduction (lines 68-76), and we have added further clarification that the dosages chosen for evaluation were picked to model the breadth of the naturally occurring range *in vivo*. See also our responses to your comment #1 and Reviewer 1's comment #2.

How was the “Ki67 index” calculated, what does it mean? It appears to simply be the percentage of Ki67 positive cells in which case it should just be called as such.

The term ‘Ki67 index’ is commonly used in pathology that does refer to the percentage of Ki67 positive cells. We replaced ‘Ki67 index’ with ‘percentage of Ki67 positive cells’.

12. Results. Figures 1,2, 4, 5, 6, 7, 8. The bar graphs should be plotted as X-Y plots because dose is a continuous variable. Additionally can the data be disaggregated and analyzed by donor cell population

(see <https://advanced.onlinelibrary.wiley.com/doi/full/10.1002/adhm.202400039>).

As explained in our response to your comment #10 above, we decided to treat with discrete doses and consider concentration as a categorical variable. Thus, we feel that the given bar charts are the ideal way to represent this data. In our opinion, desegregating data by individual donors makes the graphs onerous to read, and we are demonstrating trends within the sexes instead of individual donor differences. We decided against separating data per donor, but we have added dots to our charts showing per donor averages for enhanced insight into our data without compromising legibility of our graphics.

13. Results. Figure 3. Is there a positive control that could be used to compare these expression

levels. Additionally, what is the variance of the expression levels because the effects appear to be at most 2 fold. Were differences in expression statistically significant? The figure is also quite small consider separating it out so that it is more legible.

This data was taken from multiple cell donors across multiple cell types, and we have shown that these cells express hormone receptors (see Supplemental Figure 1). Additionally, these genes were selected from estrogen responsive gene panels found in the literature (<https://doi.org/10.1016/j.eti.2014.09.001>). As such we have not included a positive control. In addition, this data has now also been included as bar charts in Supplemental Figures 5 and 6. In these alternative formats, the variance of the data as well as statistical significance is displayed to assist in readers clarity of this data. An alternate form of the heatmaps shown in Figure 3 have been configured that arrange the graphs in a different layout to maximize legibility.

14. Results. Sex-specific effects of E2 and DHT on bulk tissue vasculogenesis. Regarding the PEG-PDMS absorption of the sex hormones (Figure S1), there is still Nile red staining with the PEG-PDMS polymer. Can the amount of absorbed Nile red be quantified so that an effective dose be determined for the sex hormones. How comparable is Nile red partitioning to that of the sex hormones (e.g., values of Kow).

We appreciate the nuance of this comment. We have added an image to Supplemental Figure 2 that shows a PDMS device not exposed to Nile Red. This added image demonstrates that the positivity seen in the clear coated PEG-PDMS device is largely background noise, and accordingly, very little Nile Red has been absorbed. Hence, we feel that it is not necessary to quantify Nile Red absorbance in the PEG-PDMS part cast in smooth molds. Nile Red partitioning relative to estradiol is now described more quantitatively with the inclusion of Kow values for both compounds. Please see lines 442-449 in the Results section.

15. Discussion. Broadly the discussion should include greater reference and contextualization with the growing literature of sex-specific cell studies for which there is an abundance for endothelial cells particularly HUVECs including,

* <https://doi.org/10.1161/01.CIR.91.3.75>

* 10.1007/s40618-014-0118-1

* <https://doi.org/10.1038/s41598-017-10139-x>

* <https://doi.org/10.1007/s10456-016-9525-6>

* <https://doi.org/10.1113/JP276048>

* 10.3390/cells10102564

* <https://doi.org/10.1016/j.mbps.2024.100145>

* <https://doi.org/10.1093/cvr/cvy185>

Thank you for pointing out these important references. We have included the suggested references and additional text that contextualizes our study in the context of the existing literature has been added to the Discussion. Please see lines 508-512.