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Relation of Flt-1 and Endothelial Function in Women Soon After Delivery: Effect of Physical Activity and Sedentary Behavior

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I. Summary

Adverse pregnancy outcomes (APOs) are believed to be caused by poor placental formation. APOs can be characterized by elevated levels of a circulating Fms-like tyrosine kinase biomarker called Flt-1, produced by the placenta when its blood vessels are shallow or inadequately formed. Flt-1 acts directly on the maternal endothelium to impair vascular function during pregnancy and contributes to maternal features of APOs. This study aimed to evaluate the relation of Flt-1 and endothelial function in women soon after delivery. This study also aimed to evaluate the relation of Flt-1 and physical activity and sedentary behavior in women during and soon after pregnancy. The processes encompassed in this study included recruitment, data collection, data organization and reduction, performance of an ELISA assay, and performance of statistical analyses. Results demonstrated sFlt-1 tended to be higher in women with versus without a history of adverse pregnancy outcomes, but there were no significant associations with Flt-1 to vascular function after delivery and circulating sFlt-1 level did not differ by current or pregnancy PA level.

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II. Abstract

Background and Significance: Vascular adverse pregnancy outcomes (APOs) are characterized by elevated levels of antiangiogenic Fms-like tyrosine kinase (Flt-1), attributable to placental ischemia. Soluble fms-like tyrosine kinase 1 (sFlt-1) is an anti-angiogenic protein detected in maternal circulation in pregnancies affected by poor placentation. Flt-1 directly impairs endothelial function during pregnancy and contributes to clinical maternal features of APOs. Associations of sFlt-1 with blood pressure (BP) and vascular function in humans soon after pregnancy have not been evaluated.

Purpose: The purpose of our study was to evaluate the relation of Flt-1 and endothelial function in women soon after delivery. The study aimed to compare serum sFlt-1 levels in women *with* versus *without* a history of adverse pregnancy outcomes, i.e. preterm birth, small-for-gestational-age delivery, preeclampsia, hypertensive disorders of pregnancy, or gestational diabetes. We evaluated associations of sFlt-1 levels and sensitive measures of vascular function soon after pregnancy. Given the angiogenic effects of exercise, we tested the hypothesis that Flt-1 would be lower in women who achieved adequate prenatal physical activity (PA) and current PA at the time of testing.

Methods: Participants delivered a singleton fetus 6 months-3 years from the time of testing, were 18-45 years old and were excluded if they smoked, had diabetes, active cancer, HIV/AIDs, or used protease inhibitors. Participants completed a blood draw, vascular testing, and completed validated surveys after an overnight fast. Vascular testing included measuring brachial BP, arterial stiffness via carotid-femoral pulse wave velocity, and reactive hyperemia (RH) was measured with venous occlusion plethysmography to quantify resistance vessel

endothelial function. A validated physical activity questionnaire (Godin Shepard Leisure-Time Exercise Questionnaire) was used to determine PA at the time of testing and second trimester pregnancy PA. APO history was determined using self-report. Serum concentration of sFlt-1 was measured using a commercially available ELISA kit. A Kruskal-Wallis test was used to detect differences in sFlt-1 concentrations; linear regression was used to evaluate associations of sFlt-1 and vascular function, adjusted for age and BMI. We tested for associations of RH with continuous levels Flt-1 using linear regression, adjusted for APO status. We used t-tests to evaluate differences in Flt-1 between women who did versus did not achieve adequate PA during pregnancy or at the time of vascular testing, adjusted for APO status.

Results: The 40 women had a mean age of 33±1 years, mean BMI of 26.3±1.0 kg/m^{2,} and 58% met adequate pregnancy PA. sFlt-1 tended to be higher in women *with* versus *without* a history of adverse pregnancy outcomes. There was no association of Flt-1 and peak RH; β=0.01±0.01, p=0.50. There was no association of Flt-1 and baseline blood flow; p>0.60. There was no difference in Flt-1 levels between women who *did* versus *did not* achieve adequate PA at the time of testing (412±17 versus 443±31 pg/ml, p=0.22) or during pregnancy (408±20 versus 430±20 pg/ml, p=0.23) and there were no significant associations when adjusted for APO. There were no significant associations sFlt-1 with BP or arterial stiffness: β=-1.03±1.2, p=0.40 for systolic BP; β=3.3±12.7, p=0.26 for arterial stiffness, even in analyses stratified by adverse pregnancy outcome status.

Conclusions: Although related to vascular dysfunction during pregnancy, Flt-1 was not related to vascular function after delivery and did not differ by current or pregnancy PA level.

Flt-1 might not be useful for identifying women at risk of vascular dysfunction after pregnancy ends.

III. Introduction

Hypertensive disorders of pregnancy and preeclampsia (collectively called adverse pregnancy outcomes [APOs]), are pregnancy induced hypertension disorders that affect both the mother and fetus. They are characterized by elevated blood pressure, with or without protein in the urine.[1] Preeclampsia can lead to eclampsia, which is one of the top five causes of maternal and infant illness and death, causing an estimated 13% of all maternal deaths worldwide.[1] Spontaneous preterm birth, which occurs in 9.7% of all births, is also a major cause of fetal morbidity and mortality and is a recognized APO.[2] APOs are believed to be caused by poor placental formation.[1,2] APOs can be characterized by elevated levels of a circulating Fms-like tyrosine kinase biomarker called Flt-1, produced by the placenta when its blood vessels are shallow or inadequately formed.[3] Flt-1 is found at high levels up to four weeks before the onset of APOs and after clinical features are present.[3] Flt-1 is a high-affinity receptor for VEGF and is expressed almost exclusively on vascular endothelial cells.[4] **Flt-1 acts directly on the maternal endothelium to impair vascular function *during* pregnancy and contributes to maternal features of APOs**.[5,6] When Flt-1 was infused into pregnant mice, hypertension developed.[5] Other studies found that vascular endothelial growth factor (VEGF) and Flt-1 were upregulated in other vascular inflammatory and proliferative disorders such as atherosclerosis and restenosis.[7] In recent years, studies have shifted interest to investigate Flt-1 function in nonpregnant women, showing that women less than two years post-pregnancy have a significant vascular store of releasable Flt-1 with no significant difference between formerly preeclamptic or uncomplicated

pregnancy.[8] **Further, infusion of Flt-1 into non-pregnant animals did not lead to hypertension or endothelial dysfunction,[5] and so, the effect of Flt-1 on vascular function soon *after* pregnancy is not known**. Given the known, direct effects of physical activity and sedentary behavior on endothelial function, it is important to test for an effect of patterns of physical activity and sedentary activity on associations of Flt-1 levels and endothelial function.[9,15] Analyzing and evaluating the role Flt-1 post-pregnancy in women could be a vital part of identifying high risk women and improving long term health outcomes for endothelial function and cardiovascular disease as higher Flt-1 values may be associated with angiogenesis, vascular inflammatory disorders, and atherosclerosis.

In previous literature, it has been unclear whether an APO causes cardiovascular disease (CVD), or whether APOs and CVD share common risk factors and an APO is a symptom of someone destined to have eventual CVD.[1] We know that there is a significant association between having an APO such as preeclampsia and the development of hypertension after pregnancy.[16] We also know certain biomarkers identify preeclampsia.[18,20] If the two, preeclampsia and CVD both share similar risk factors, it is logical to try to determine whether there are similar biomarker patterns useful in identifying the onset of these diseases. Because Flt-1 has been confirmed as a biomarker for placental dysfunction with usefulness for identifying high-risk pregnancies for preeclampsia and fetal growth restriction with placental involvement,[18] we must look at the possibility that Flt-1 could be useful for identifying future intermediate cardiovascular health outcomes soon after pregnancy. We asked the question: will higher levels of Flt-1 be associated with poorer measurements of cardiovascular health after pregnancy, such as BP, arterial stiffness, and reactive hyperemia?

In studies that have evaluated preeclamptic, gestational hypertensive, and normotensive

pregnant women to compare various statuses of self-reported physical activity, results showed

that increasing levels of leisure-time physical activity (LTPA) were associated with a reduced

risk of preeclampsia.[17] Furthermore, increasing the amount of time spent active each day was

associated with a decreased risk for preeclampsia, and increasing amount of time spent sitting

per day was associated with an increased risk of preeclampsia.[17] Because it has been proven that

preeclampsia is associated with elevated circulating Flt-1 protein,[5, 8, 18] and preeclampsia has

been inversely associated with PA,[17] we must ask will levels of Flt-1 be associated with levels of

PA in women *with* and *without* APOs?

Asking this question expands on current studies that have examined the protective effects

of exercise during pregnancy in mouse models.[19] Findings showed reduced circulating and

placental soluble Fms-like tyrosine kinase-1 in trained transgenic mice (mice with wheels in

cages for exercise) compared with sedentary mice.[19] With decreased levels of sFlt-1 in active

mice, it is evident that exercise may have a protective effect in preventing the development of

preeclampsia ins animal models.[19] These findings suggest the need to further expand these

results and analyze the possible protective effects of exercise with Flt-1 levels in human models.

Previous research shows that nonpregnant patients with chronic kidney disease (CKD)

have an increased risk of heart failure.[14] When studying Flt-1 in this CKD patient population,

results showed that there was a direct association between Flt-1 and heart failure.[14] Following

this trend, we predict that other individuals (outside of this CKD patient population) may also

show higher levels of sFlt-1 with heart failure. It is necessary to analyze the relationship between

Flt-1 and CVD in patients outside this specific CKD population to produce more generalizable results.

In addition to its function in vascular endothelial cells and placental trophoblasts, Flt-1 is a useful cell surface marker for monocyte-macrophages in humans.[4] Although the arterial wall contains a large number of resident macrophages and some resident dendritic cells, studies have shown that atherosclerosis drives a rapid influx of inflammatory monocytes and other patrolling monocytes.[21] Once present in the vessel wall, monocytes differentiate to a phenotype consistent with inflammatory macrophages and inflammatory dendritic cells.[21] This evidence prompts that elevated Flt-1 levels in patients may identify the elevated monocyte and macrophage influx characteristic of CVD.

On a wider scope, cardiovascular disease (CVD) is the number one cause of mortality in women in the United States and developing countries.[22] More specifically, CVD is the second leading cause of death for all women in South Carolina and is the leading cause of death for African American women in South Carolina.[23] Localized numbers on the issue in South Carolina reflect that in 2013, 4,351 women died from CVD, there were 23,065 hospitalizations associated with CVD, and there were over 1.24 billion dollars spent on hospitalizations due to CVD in the state.[23] CVD in women is an urgent issue that needs to be addressed from multiple angles, looking at individual biological, social, economic, and behavioral factors. From my time spent in Dr. Lane-Cordova's Women's Vascular Health lab at USC, I have broadened my understanding of how adverse pregnancy outcomes (APOs) are major, previously underappreciated risk factors for CVD in women after pregnancy. In fact, CVD risk is 1.8- to 4.0-fold higher in women who have had an adverse pregnancy outcome.[24] Understanding how these APOs progress to CVD for

women within our state of S.C. and further the USA as a whole is absolutely critical to developing strategies aimed at reducing CVD risk long term for mothers.

Methodology

The purpose of my project was to evaluate the relation of Flt-1 to endothelial function in women 6 months to 3 years post-pregnancy, as well as relate these values categorically to self-reported physical activity or sedentary lifestyle. I aimed to answer the following questions:

1. a) is Flt-1 higher in women who had an APO in the first 3 years after pregnancy?

2. b)Is Flt-1 correlated to endothelial function in the first 3 years after pregnancy?

3. c) Is Flt-1 correlated with physical activity or sedentary lifestyle after delivery?

4. d) Does APO status modify correlations of Flt-1 and endothelial function?

In this ancillary to Dr. Lane-Cordova's ongoing case-control study conducted in women who had a singleton pregnancy within the past 6 months-3 years, I prosed an additional Flt-1 assay to the established data collection/analysis. The additional assay did not affect participant burden. We had already collected complete data from 19 women prior to the beginning of my project. We continued to collect data throughout the year 2019- 2020 year and performed the ELISA assay with a sample size of 40 total women in early February 2020.

IV. Materials and Methods

All women were tested during the follicular phase of their menstrual cycle, had fasted and avoided caffeine for a minimum of 8 hours, and refrained from taking anti-inflammatory medications for 3 days prior to the study visit. After obtaining informed consent, venipuncture was used to obtain a blood sample. Participant height and weight were recorded before resting 5

minutes quietly in a dimly lit room. We measured blood pressure readings using an automated

oscillometric cuff (Omron Medical, Japan), performed vascular tests, and obtained additional

information with validated surveys.

The following tests and techniques were used to evaluate the quantity and function of

FLT-1 in participants:

Biomarker Assays*:* Blood was drawn before the vascular tests after an overnight fast to

determine levels of circulating Flt-1 using ELISA assays (R & D Systems, Minneapolis, MN).

The standards and duplicate participant serum blood samples were loaded onto a 96 well

microplate coated with a monoclonal antibody specific for human VEGF R1 (Flt-1). After

completion of the assay, the duplicate readings for each standard and sample were averaged. A

standard curve was created by reducing the data (Figure 1).

Arterial Stiffness*:* The distance between the suprasternal notch and the femoral and carotid

arteries was measured to determine the pulse wave velocity (PWV) distance. PWV was then

calculated as m/s using the distances and the time delay, in seconds, between 10 proximal and

distal waveforms as determined from simultaneous ECG tracings, which is the gold-standard

measurement technique for quantifying arterial stiffness.[10]

Strain gauge plethysmography: Reactive hyperemia (RH) and forearm blood flow, surrogates

for microvascular endothelial function, were measured using a dedicated system with built-in

analysis software (Hokansen, Bellvue, WA). The forearm was measured and fitted with the

proper strain gauge. A blood pressure cuff was wrapped around the wrist and two cuffs fitted

around the upper arm. The strain gauge was fastened around the widest part of the forearm. The

wrist cuff was rapidly inflated to obstruct hand blood flow. The first upper arm cuff was then

automatically inflated for 7s to prevent venous backflow while forearm volume was measured

via the strain gauge. The cuff was deflated for 8s. This cycle was repeated 6 times and averaged

to determine resting forearm blood flow. To determine vascular reactivity, the second upper arm

blood pressure cuff was inflated to 250 mmHg for 5 minutes. At 4 minutes, one minute before

deflating the upper arm cuff, the wrist cuff was inflated. The upper arm cuff was then quickly

deflated, and the strain gauge recorded changes in forearm volume for 3 minutes using the same

cycling to prevent venous backflow during measurements as described above. Peak blood flow,

i.e., the increase in forearm volume in the first 15 seconds, and total reactive hyperemia (area

under the curve) throughout the 3-minute period were recorded.[11] For this analysis, we set peak

blood flow as our measure of endothelial function.

Physical Activity and Sedentary Behavior: Participants filled out a validated physical activity

questionnaire and other surveys to determine their pregnancy and medical history,

sociodemographic information, sodium content of diet, and sedentary behavior.[12,13]

Statistical Analyses: In order to analyze the data collected, a Kruskal-Wallis test was used to

detect differences in sFlt-1 concentrations; linear regression was used to evaluate associations of

sFlt-1 and vascular function (BP and PWV for arterial stiffness), adjusted for age and BMI. We

tested for associations of RH with continuous levels Flt-1 using linear regression, adjusted for

APO status. We used t-tests of equal variances to evaluate differences in Flt-1 between women

who *did* versus *did not* achieve adequate PA during pregnancy or at the time of vascular testing.

Analyses were performed on STATA version 14.0 (Stata Corp. College Station, TX). We set our

alpha at 0.05. Our measurements of PWV for arterial stiffness as well as administration of the

Godin-Shepard Leisure-time Physical Activity Questionnaire (GSLTPAQ) were chosen because

they are the validated and gold standard techniques to measure leisure time physical activity and arterial stiffness.[10,13]

**Certifications:** Before beginning this project, I completed CITI human subjects research training and general biosafety training at The University of South Carolina. After this training and being added to the IRB, I participated in a training period led by Dr. Lane-Cordova to master performing the measurements in lab. I then began participation in data collection for the core study described above. Amendments have been made as necessary to the IRB when adding new methodology and personnel to the lab team and study.

V. Expected Results and Significance

While current studies demonstrate that Flt-1 directly impairs endothelial function during pregnancy, there is scarce understanding of the relationship between Flt-1 and endothelial function in women post-pregnancy. Thus, the value of Flt-1 as a biomarker of vascular dysfunction in humans soon after pregnancy is not clear. Little is known regarding the relation of Flt-1 to regular physical activity and sedentary behavior. My project provides preliminary evidence regarding associations of Flt-1 levels with vascular function, independent of and dependent on physical activity behaviors, in women soon after pregnancy. I originally anticipated that levels of Flt-1 would be inversely related to endothelial function and directly related to arterial stiffness. Furthermore, I anticipated that sedentary behavior would be directly related, and physical activity will be inversely related to Flt-1 levels. I plan to share the results of this study with all participants along with a brief summary of the conclusions we have made. I also originally planned to present at Discover USC in Spring 2020 as well as present at The

American College of Sports Medicine national conference (ACSM; San Francisco, CA) in 2020, however, this was pre-COVID-19 regulations and cancellations. A portion of this thesis was submitted for presentation at ACSM. I am currently in the process of preparing a manuscript based on this study for publication in obstetrics/lifestyle medicine journals.

VI. Results

Participants. Of the 40 women, the mean age was 33±1 years and mean BMI was. 27.3±1.0 kg/m^2 (Table 1). When analyzing using a Kruskal-Wallis test for skewed data**,** sFlt-1 levels tended to be higher in women who had a history of an adverse pregnancy outcome, $p<0.10$.

Physical Activity. A score of at least a 24 on the Godin-Shepard Leisure-time Physical Activity Questionnaire (GSLTPAQ) is defined as sufficiently active*.* Results from the GSLTPAQ showed that 33 women had a status of sufficiently active at the time of testing, and 7 did not (Table 1). When analyzing using two sample t-tests with equal variances, there was no significant difference in Flt-1 levels between women who did versus did not achieve adequate PA at the time of testing (412±17 versus 443±31 pg/ml, $p=0.22$). Results from the GSLTPAQ demonstrated that 23 women had a status of sufficiently active during their second trimester of pregnancy and 17 did not (Table 1). When analyzing using two-sample t-tests with equal variances there was no significant difference in Flt-1 levels between women who did versus did not achieve adequate PA during pregnancy (408±20 versus 430±20 pg/ml, $p=0.23$).

Regression. There was no association of Flt-1 and peak reactive hyperemia (RH) β=0.01±0.01, $p=0.50$ (Figure 2:A). There was no association of Flt-1 and baseline blood flow, $p>0.60$.   There were no significant associations of sFlt-1 with blood pressure (BP) or pulse wave velocity

(PWV) measuring arterial stiffness: $\beta=-1.03\pm1.2$, p=0.40 for systolic BP; $\beta=3.3\pm12.7$, p=0.26 for PWV, even in analyses stratified by adverse pregnancy outcome (APO) status (Figure 3: A,B). Further, there were no significant associations of pregnancy PA or PA at the time of testing with sFLt-1 levels when adjusted for APO status with linear regression.

VII. Discussion

In this study, we expanded on the current literature aimed to evaluate the effect of Flt-1 on vascular function soon after pregnancy. Our study results support the current literature in place previously conducted in mouse models that Flt-1 may not be a predictor of endothelial dysfunction soon after pregnancy.[5] With our results showing no association between sFlt-1 level and vascular function in women soon after pregnancy, in combination with the previous studies which demonstrate the same in mouse models after pregnancy, we conclude that Flt-1 may not be a biomarker that predicts vascular function and cardiovascular outcomes in women soon after pregnancy. Our body of research is the first preliminary body of data that supports this realization for the human population.

Furthermore, although PA has shown an inverse relationship with preeclampsia in previous literature,[17] in our study, PA reported from the second trimester of pregnancy and PA reported soon after pregnancy at the time of testing showed no association with Flt-1 levels. The time frame our study focuses on for PA presents a limitation that should be addressed in the future. The limitation prompts us to evaluate the level of PA before pregnancy and during the beginning of pregnancy to expand on the current literature within mouse models. The question we hypothesized in our study "Will levels of Flt-1 be associated with levels of PA?" may be

more beneficial to ask when PA is evaluated in the first trimester of pregnancy and even soon

before pregnancy in women. Future studies should aim to expand on the current mouse model

research that PA decreases levels of sFlt-1 during pregnancy,[17] as this protective effect should be

evaluated in humans. There is still a gap in this portion of literature and room to analyze the

possible protective effects of PA on Flt-1 status before and during the beginning of pregnancy

for women.

　　We know that women who have APOs including preeclampsia are at a higher risk for

CVD.[24] Our results confirm our hypothesis that women who have had APOs will have higher

levels of sFlt-1 after pregnancy. While our population focused on younger females soon after

pregnancy whose vascular outcomes did not show association with Flt-1 levels, it may be

beneficial to follow these specific individuals for years to come to analyze long term CVD

outcomes. We discussed two bodies of previous research that demonstrate the rationale for

evaluation of these outcomes: the first being the study of the population of non-pregnant CKD

patients whose Flt-1 levels showed a direct association with heart failure and the second being

the evidence that elevated Flt-1 levels are associated with monocyte and macrophage influx

(characteristic of atherosclerosis).[14,21] Our study limits the scope of assessing vascular function

and Flt-1 to women soon after pregnancy, but future studies should evaluate levels of Flt-1 for

women whose health statuses indicate risk for CVD in years later down the road. It would be

interesting to longitudinally follow women from our study to analyze Flt-1 levels in the women

with and *without* APOs, gathering data each decade to see if associations in Flt-1 change based

on future CVD incidence. We would predict that women with APOs would have higher

incidence of CVD in the long-term future, and a higher Flt-1 levels may accompany the onset of

CVD as discussed in the two bodies of previous research referenced. If so, Flt-1 levels could be evaluated in future patient populations to identify the early onset of CVD, and thus clinically aim to adjust health statuses for women before they become morbidly disabling or fatal.

VIII. References

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IX. Tables and Figures

<u>**Table 1:**</u> **Participant Characteristics (n=40)**

Characteristic	Mean ± SE
Age (yrs)	33.1 ± 0.78
Adverse Pregnancy Outcome (%)	40
BMI (kg/m^2)	27.3 ± 1.05
BSP (mmHg)	109 ± 2.1
BDP (mmHg)	70.2 ± 1.6
ASP (mmHg)	98.5 ± 1.9
ADP (mmHg)	70.9 ± 1.6
PWV (m/s)	6.06 ± 0.18
Pregnancy PA (GSLTPAQ score)	32.3 ± 3.9
Current PA (GSLTPAQ score)	43.5 ± 4.4
Pregnancy Weekday Sedentary Time (hrs/day)	9.5 ± 0.7
Current Weekday Sedentary Time (hrs/day)	8.5 ± .7

Table Legend: BMI: body mass index; BSP: brachial systolic pressure; BDP: brachial diastolic pressure; ASP: aortic systolic pressure; ADP: aortic diastolic pressure; PWV: pulse wave velocity, GSLTPAQ: Godin-Shepard Leisure time Physical Activity Questionnaire Score. The reported values are an exercise unit score from the validated survey. Sufficiently active is defined by a Godin score of at least 24.
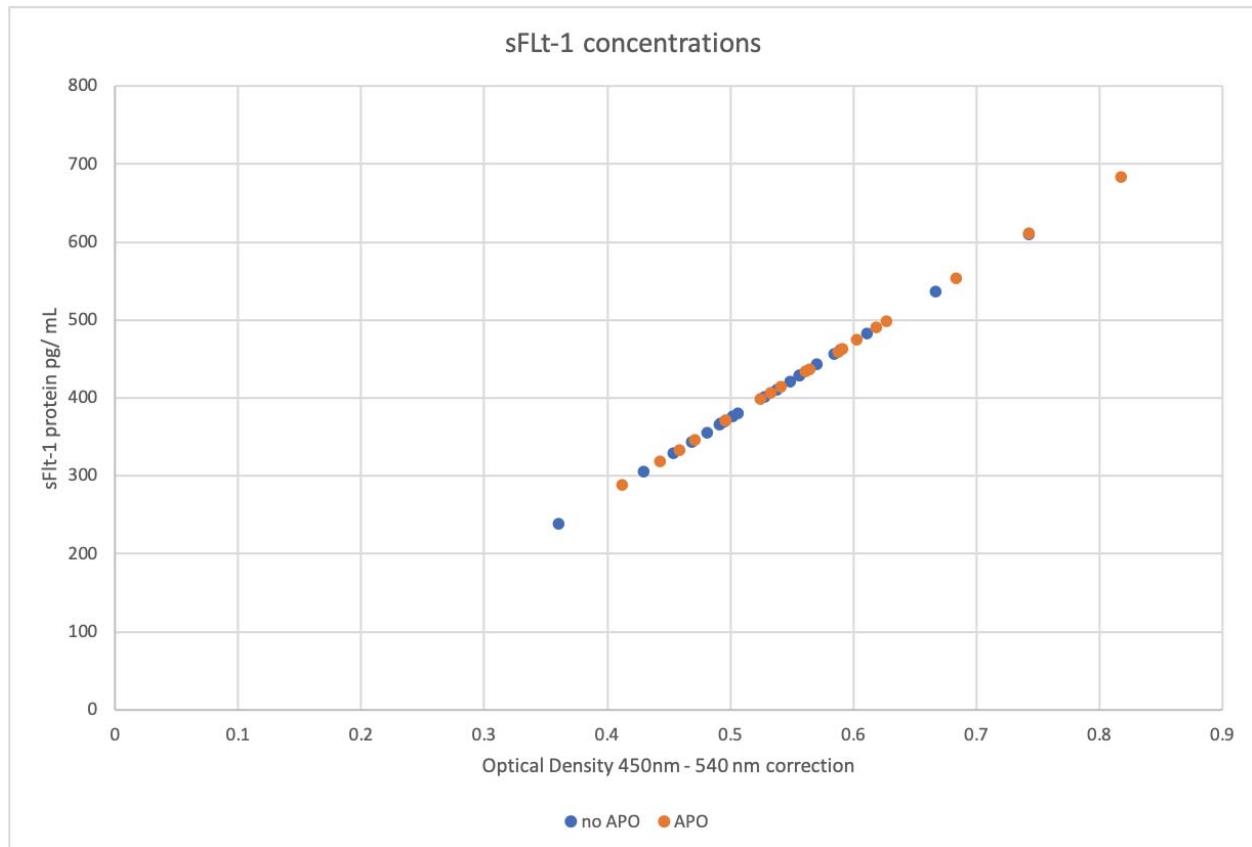
Figure 1. sFlt-1 levels based on APO status

Figure 1 Legend. APO: Adverse Pregnancy Outcome.

sFlt-1 levels are significantly higher for women who have had an APO, $p < 0.10$.

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<u>Figure 2: A)</u> Peak RH and <u>B)</u> Baseline Blood flow and sFlt-1 levels in women with and without a history of adverse pregnancy outcomes.
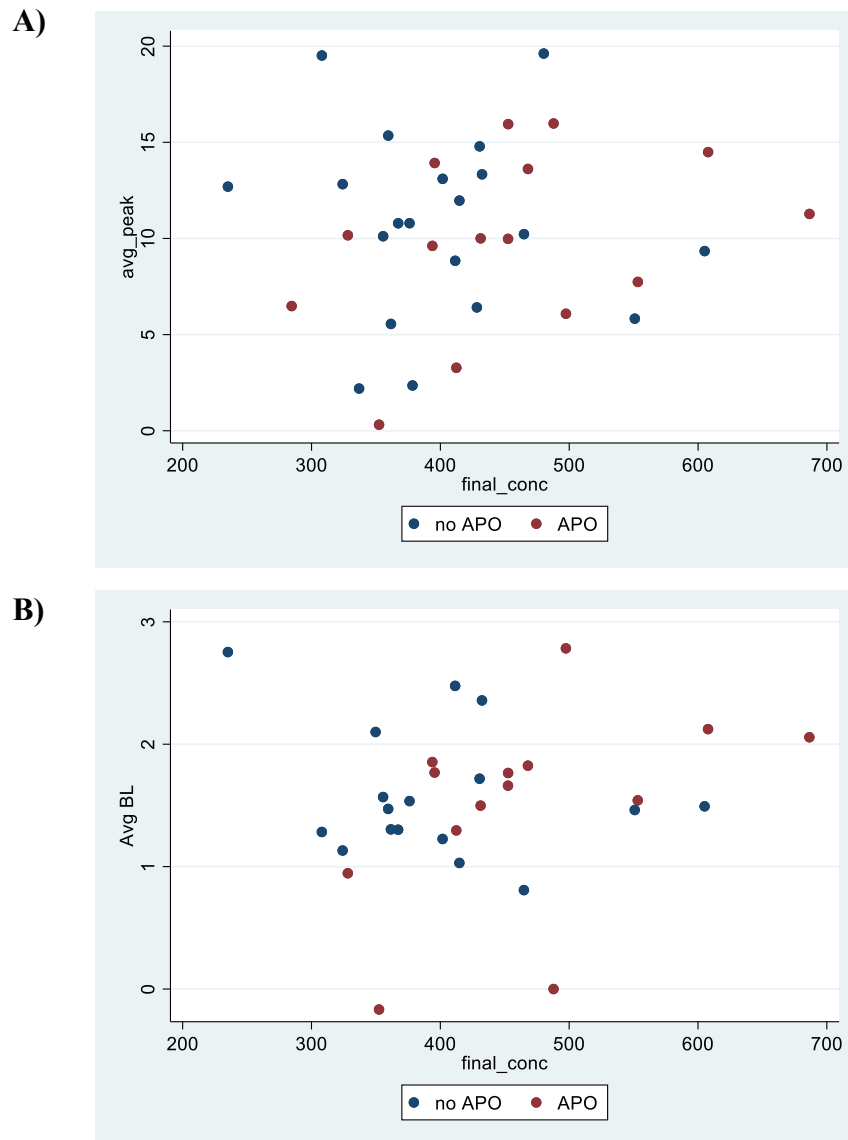
A)

B)

Figure 2 Legend. APO: Adverse Pregnancy Outcome

There is no association with Flt-1 level and reactive hyperemia or baseline blood flow, even when adjusted for APO status.

Figure 3: A) BSP and B) PWV and sFlt-1 levels in women with and without a history of adverse pregnancy outcomes.
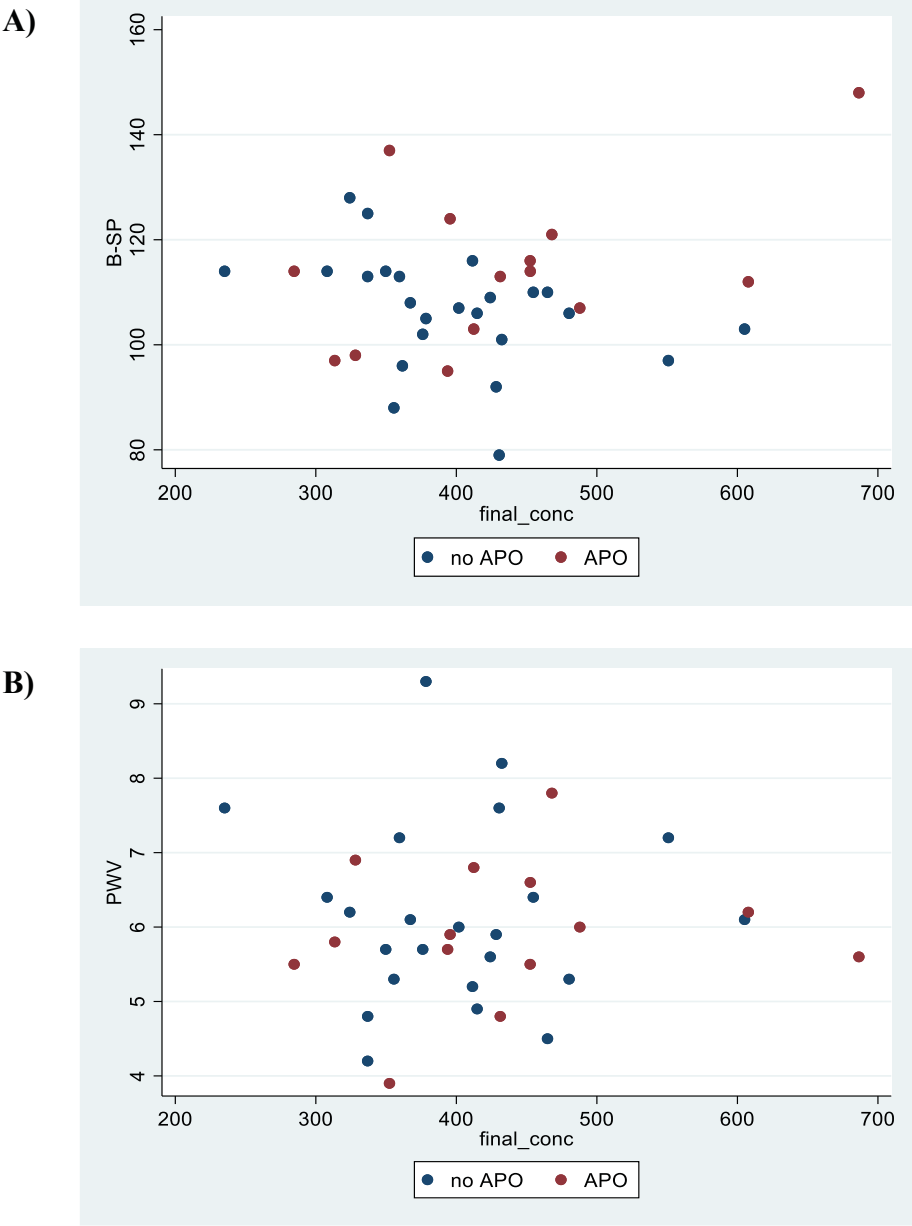
A)

B)

Figure 3 Legend. APO: Adverse Pregnancy Outcome

There is no association with Flt-1 level and BSP or PWV, even when adjusted for APO status.

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