Study Title: Dietary supplements to improve vascular inflammation after an adverse pregnancy outcome

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A. SPECIFIC AIMS

1. **Aim 1. Determine the effects of a 4-week course of anti-inflammatory supplementation (PolyResveratrol-SR®) on vascular function, subclinical atherosclerosis and CV biomarkers**

2. **Aim 2. Define adherence and barriers to compliance to supplementation.**

B. BACKGROUND AND SIGNIFICANCE

Adverse pregnancy outcomes (APOs) occur in up to 20% of all pregnancies and are associated with a lifetime of higher cardiovascular disease (CVD) risk[1,2]. **Aberrant and persistent systemic inflammation is a hallmark of both APOs and CVD.**[3] An inappropriate uterine innate immune response to invading fetal cytotrophoblasts is thought to underlie poor placentation and vascular-related APOs; inflammation of the uterine cavity evokes contractions and contributes to preterm birth.[4] Levels of cytokines involved in endothelial inflammation, an instigating event in hypertensive and atherosclerotic processes, were higher in the 2nd trimester in women destined to have APOs.[5] Women with a past APO have higher levels of systemic inflammation, including c-reactive protein and interleukin-6 (IL-6), in the years after delivery versus women with no APOs. A similar pro-inflammatory cytokine profile has been observed in patients with overt CVD. **Intervening on systemic inflammation may be a viable strategy to interrupt the progression from APO to CVD.**

Dietary supplements, such as polyphenols, are anti-inflammatory and inhibit vascular inflammation with potentially few barriers to adherence. **Given the hypothesized contribution of a persistent pro-inflammatory state to the excess CVD risk observed in women after APO, utilization of a polyphenol dietary supplement to specifically target inflammation holds promise as a low-burden intervention to prevent or delay APO-related CVD.** To our knowledge, efficacy and tolerability of a dietary polyphenol supplement has not been investigated in women with a history of APO. To fill in this critical gap in knowledge, we propose to test the vascular function and CV biomarker, i.e., IL-6, IL-10, intracellular and vascular cell adhesion molecule-1 (ICAM-1 and VCAM-1), responses to a 4-week anti-inflammatory dietary supplementation in a clinical trial pilot study of 56 women, 28 assigned to the intervention and 28 to the control group, in the first 3 years after an APO.

C. PRELIMINARY STUDIES

Other studies of endothelial dysfunction and APOs have consistently shown that women who go on to have APOs have evidence of endothelial dysfunction that is detectable in the 1st trimester of pregnancy[5]. In a recent paper, we demonstrated that there was no difference in evidence of pre-pregnancy endothelial dysfunction between women who did and did not go on to have a preterm or small for gestational age delivery ~3 years later (OR: 1.01, 95% CI: 0.74, 1.39). Our results suggest that the endothelial dysfunction that contributes to APOs (and eventual CVD) may not develop until pregnancy begins, highlighting the importance of the pregnancy period for influencing the CVD risk trajectory in women. In this investigation, only black race was associated with greater risk of preterm birth and/or small for gestational age delivery, even after adjustment for relevant covariates

Higher BMI and central BP in women with a past APO. In early data from an ongoing study conducted in the PI's lab, we found a trend towards higher central systolic BP in women with a past APO (*Table, unpublished data*). Findings suggest that differences in sensitive measures of vascular function are detectable soon after an APO but before overt CVD.

	APO+ n=17	APO- n=36
Age (yrs)	33	33
Race (% AA)*	28	17
aSBP (mmHg)*	102	97
bSBP(mmHg)	113	109
BMI (kg/m^2)*	29.2	26.0

Table Legend. AA: African American; aSBP: aortic systolic BP; bSBP: brachial systolic BP

D. RESEARCH DESIGN AND METHODS AND DATA ANALYSIS

Research Design. We will invite 56 women with an APO within the past 3 yrs to participate. Women will be randomly assigned to the 4-wk intervention group (n=28) or a standard care group (n=28) using a computerized random number generator, stratified by race. Assuming up to 25% with poor adherence, we expect that 42 women (21 each arm) will complete the study. No data exists related to effects of supplementation on vascular function in our population, we assumed a medium effect size, i.e. Cohen's d~0.50 and accepting a β=0.80, to generate sample sizes. Generation of effect sizes is a key outcome of this study. We will conduct a vascular assessment and blood draw at two visits: before the intervention begins and within 3 days of completing the intervention. We will conduct visits during the early follicular phase of menstrual cycle to account for fluctuating hormones. Vascular testing will be performed in the supine position and ≥4 hours after a light meal. Surveys for Aim 2 will be completed within the first 2 weeks of study enrollment, and interviews for Aim 2 will be conducted within 1 week of Visit 2, in-person or via telephone.

Anti-inflammatory supplementation intervention. Participants randomized to the supplementation intervention will receive PolyResveratrol-SR® (100 mg curcumin phytosome, 100 mg quercetin phytosome, 100 mg green tea phytosome, 100 mg trans-resveratrol, 100 mg trans-pterostilbene; Thorne Research). Participants will be asked to take 2 doses/day (1g total). Participants randomized to supplementation will receive a daily email reminding them to take the supplement. **Covariates.** Medical history, age, and sociodemographics will be obtained via self-report. We will use validated surveys to determine physical activity, sedentary behavior, and smoking history. Height and weight will be measured.

Aim 1 Outcomes. The outcomes will be measured in-person. All outcomes are listed and described below. Dr. Lane-Cordova has extensive experience collecting the measurements described below.

1. *Blood pressure*. Resting brachial systolic BP (SBP) and diastolic BP (DBP) will be measured using an automated oscillometric cuff (HEM-907 XL; Omron, Shimane, Japan) after 10 min of supine rest in a quiet, dimly lit room. Brachial BP will be obtained in duplicate with 1 min between the first and second reading. If the two values are not within 5 mmHg of each other, another measurement was taken until two consecutive values within 5 mmHg of each other are obtained. These two values will be averaged and used for analysis.

2. *Arterial stiffness*. Participants will remain supine in a quiet room. The aortic waveform will be reconstructed from radial artery pressure waveforms obtained from a 10-s epoch

using applanation tonometry (Millar Instruments, Houston, TX) and calibrated with brachial MAP and diastolic BP with a generalized validated transfer function[6] (SphygmoCor; AtCor Medical, Sydney, Australia) From this central waveform, we will derive central BP, augmentation index (AIx), and end-systolic pressure. An in-device quality rating of ≥80 % will be required for all recordings used in analysis. We will measure central (aortic) pulse wave velocities in the supine position using gold-standard techniques[7]. We will measure distances from the suprasternal notch to the femoral artery and from the carotid artery to the suprasternal notch with a tape measure, recorded to the nearest mm. The distance from the carotid site to the suprasternal notch will be subtracted from the distance between the suprasternal notch and femoral artery to account for the divergent directions of pulse wave propagation. We will use the same high-fidelity strain-gauge transducer to obtain pressure waveforms at the right common carotid artery and then at the right femoral artery for central pulse wave velocity. Pulse wave velocity will be calculated as distance/time, using the distances between measurement points and the measured time delay between 10 proximal and distal waveforms (SphygmaCor; AtCor Medical). ECG will be recorded simultaneously, and the peak of the R wave will be our timing marker. BP influences pulse wave velocity, so we will also adjust pulse wave velocity for aortic MAP.

3. *Resting forearm blood flow*. Forearm blood flow (FBF) will be measured using strain gauge plethysmography (EC-4; Hokanson, Inc., Bellevue, WA). A standard BP cuff will be positioned around the upper arm. An appropriately sized strain gauge will be placed around the widest part of the forearm, and a pediatric cuff placed around the wrist to occlude hand blood flow. Before measuring FBF, the wrist cuff will be inflated to 250 mm Hg of pressure for 1 minute. FBF will be determined by inflating the upper cuff to 50 mm Hg (to oppose venous backflow) for 7 s and then deflating it for 8 s. An average of six of these 15-s plethysmographic cycles will be used to determine FBF, expressed as milliliters per minute per 100 mL of forearm tissue and as flow per unit pressure, e.g. conductance, using the equation: *conductance = (FBF/MAP) x1000*.
4. *Vascular endothelial function*. Reactive hyperemia, e.g. resistance vessel endothelial function, will be measured immediately after FBF. Arm blood flow will be occluded by inflating the upper arm BP cuff to 250 mm Hg for 5 min. One minute prior to the release of the upper arm cuff, the wrist cuff will also be inflated to 250 mm Hg. The upper arm cuff pressure will be rapidly released and changes in forearm volume will be recorded via the strain gauge, in 15-s cycles as described above, for 3 min (13 readings)[8]. The highest reading will represent peak blood flow. All 13 measurements will be plotted against time, and the area under this curve will represent total reactive hyperemia[8]. We chose to measure reactive hyperemia rather than flow-mediated dilation due to the high intra- and interobserver variability in flow-mediated dilation measurements[9].
5. *Carotid intima-media thickness*. The right common carotid artery will be imaged with ultrasound (Logiq GE S8) using a 12-MHz linear array probe. Intima-medial thickness will be defined as the distance between the leading edge of the lumen–intima border and the leading edge of the media–adventitia border on the far wall of the artery[10]. We will average a 5mm segment obtained approximately 20 mm proximal to the carotid bifurcation for analysis.
6. *Blood analyses*. We will collect 30 mL(2 Tb) of blood into an EDTA containing tube and serum tube using venipuncture and a butterfly needle. Blood will be centrifuged at 1300 g for 10 min, aliquoted into 0.25 mL cryotubes, and stored at -80ºC until analysis. We will determine levels of circulating IL-6, IL-10, ICAM-1, and VCAM-1 using commercially available ELISA kits (R&D Systems). We will bank unusedl blood for future, as yet undetermined analyses. Participants will be informed of this intent before providing blood. Blood will be labelled and linked to clinical data and demographics using a

unique identifier; samples will be de-identified for storage at the PHRC and for future analyses.

7. *Covariates: Medical and Pregnancy History and Demographics*. We will ask questions about demographic, medical, and pregnancy history. Answering questions will not provide identifiable information, *Self-reported activity:* We will use validated surveys to quantify physical activity and sedentary behavior,[12,13] *Diet History.* We will use a validated survey to assess diet composition in the previous 6 months.[14]

Data Analyses. We will calculate the 25th and 75th percentile for each biomarker and vascular function test and use these values as cut-offs for "low" and "high" levels, respectively. We will test for differences in biomarker levels between women by APO status and race using a t-test or Mann-Whitney U test, depending on the distribution of the variables. We will use separate multiple linear regression models to determine associations of each exposure (continuous levels of biomarkers; quartiles; and high or low levels of these biomarkers) with continuous measures of our outcomes (individual indices of vascular function, e.g. BP, arterial stiffness, augmentation index, FBF, endothelial function, and intima-media thickness) in all 56 women. We will use logistic regression to test for associations between continuous and categorical levels of biomarkers with categorical outcomes: the likelihood of having hypertension or prehypertension, high central arterial stiffness, low endothelial function, and high carotid intima-media thickness in all 56 women. We will test for unadjusted associations and then adjust all regression analyses for key co-variables: maternal age, race, and smoking. We will test for associations after furthering adjusting for years of education, body mass index (BMI), parity, and physical activity, in the index pregnancy in exploratory analyses. We will test for an interactive effect of APO status and race on these associations using a multiplicative interaction term in separate analyses and conduct all analyses stratified by APO status and/or race if the interaction is significant. We will conduct a sensitivity analysis by repeating our statistical tests only in the primiparous women as prior pregnancy is associated with lasting changes in vascular function and may influence our outcome and exposure variables.

Aim 2 Outcomes:

1. *Acceptability of Dietary Supplementation.* We will administer surveys to all participants to understand their perceptions of dietary supplementation, including potential benefits and consequences associated with supplementation. We will determine willingness to use supplements, barriers to supplementation, and who, i.e., physician or partner, influences their decision to use dietary supplements.
2. *Adherence and Barriers to Adherence.* We will count pills to determine adherence to supplementation regime. We will assess each intervention participant's perceptions of supplementation with a brief interview at the final visit. Interviews will capture participant's perceptions of the role of supplementation for CVD prevention, and any side effects of supplementation. We will ask participants to recommend ways to encourage participation and ask questions regarding their perceptions of the incentive structure and contact with the study team. We will contact participants who drop out of the study to identify reasons for drop out.

Statistical analyses: Acceptability survey data will be collected using a Likert Scale, tallied and averaged. We will determine the proportion of participants with adequate adherence (≥80%) to supplementation. If ≥80% of women have adequate adherence, we will conclude acceptable adherence is feasible with the existing incentive structure. Analysis of all post-intervention interview data will use an inductive approach and systematically guided by the constant

comparison technique. Qualitative data analysis software (QRS NVivo 10) will be used to code data and organize the analysis.

E. PROTECTION OF HUMAN SUBJECTS

1. TARGET POPULATION:

Participants and incentives. We will invite women who had a vascular APO 6 months-3 years ago[15] and were 18 years or older at the time of the APO, to participate. Exclusion criteria: currently pregnant or breastfeeding, current smoking, active cancer, regular use of NSAIDs, steroidal medications, statins, or other anti-inflammatory supplements, HIV/AIDS, uncontrolled high blood pressure, unwilling or unable to use a dietary supplement, known sensitivity to resveratrol, curcumin, green tea, or quercetin. We will provide a cash incentive of $50 after study Visit 1 and Visit 2. If enrollment numbers are below target at 6-months, we will open enrollment to women with a history of gestational diabetes (GDM) as GDM is also associated with inflammation and excess CVD risk in women. We chose the time interval of 6 months to 3 years after delivery because it takes about 6 months to return a pre-pregnancy physiological state, but vascular dysfunction has been documented for at least 3 years after an APO[15].

2. RECRUITMENT PLANS:

We will target recruitment channels including: 1) promotions in publications with low or no advertising cost (i.e., Craig's List); 2) electronic and social media recruitment blurbs (i.e., Facebook, Twitter, Instagram, website, email newsletter, electronic billboard, etc.); 3) posting flyers around USC campus area and local Columbia businesses and health centers; 4) word of mouth, 5) ads in local papers and place flyers in public places.

Additionally, patients will be recruited from Prisma USC Obstetrics and Gynecology Center (2 Medical Park, Columbia, SC). Prisma staff may identify potentially eligible participants and direct them to the lab website or to talk to research staff for more information about the study.

All recruitment material will list study eligibility criteria and requirements and include a phone number and email address where participants can reach staff if they have any questions regarding study participation. Potential participants will be screened over the phone by a research assistant and scheduled for a visit if they meet requirements.

3. EXISTING DATA/SAMPLES:

Not applicable

4. CONSENT/ASSENT:

Participants fill complete a written, informed consent form prior to participation in the study.

5. POTENTIAL RISKS:

The risks of the proposed studies are minimal. The main risks are (1) minor discomfort or bruising during phlebotomy and plethysmography; (2) unforeseen side effects of supplementation; and (3) loss of confidentiality. We will minimize risks 1 and 3 with standardized training of all study staff and by using HIPAA-compliant databases. Participants will be seated during the blood draw, and juice and snacks provided if needed. A maximum of three needle sticks will be permitted. Given that the supplement for the study is readily available over the counter and we're screening for known sensitivity, we do not anticipate severe side effects. We

will inform participants of the supplement ingredients during screening and in the Informed Consent document and exclude individuals with known sensitivity to the ingredients.

6. POTENTIAL BENEFITS:
Participants will not experience any benefits from participating in this study.

7. CONFIDENTIALITY
All of participants data will be deidentified. Only study team members will have access to participant information and study data. All data will be uploaded from these secure sites by study personnel and saved on the department server. These data are stored behind an encrypted firewall, and automatically backed up. All data will be stored at least 7 years after the completion of the study.

8. COMPENSATION:
Participants will be compensated $50 at both study visits. We will pay for parking at our lab.

9. WITHDRAWAL:
Participants will be informed they can leave the research at any time without penalty. If a participant decides to withdraw, no more information will be collected.

Any data collected during participation in this research study prior to the date the participant chooses to withdraw consent may continue to be used by the investigators for the purposes described above.

Choosing not to be in the study will not result in any penalty or loss of benefit to which participants are entitled. Specifically, the choice not to be in this study will not negatively affect a participant's right to any present of future medical treatment or his/her present or future employment (for employees at USC or its affiliates).

F. REFERENCES/LITERATURE CITATIONS

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