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Vitamin C supplementation improves placental function and alters placental gene expression in smokers

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Maternal smoking during pregnancy (MSDP), driven by nicotine crossing the placenta, causes lifelong decreases in offspring pulmonary function and vitamin C supplementation during pregnancy prevents some of those changes. We have also shown in animal models of prenatal nicotine exposure that vitamin C supplementation during pregnancy improves placental function. In this study we examined whether vitamin C supplementation mitigates the effects of MSDP on placental structure, function, and gene expression in pregnant human smokers. Doppler ultrasound was performed in a subset of 55 pregnant smokers participating in the "Vitamin C to Decrease the Effects of Smoking in Pregnancy on Infant Lung Function" (VCSIP) randomized clinical trial (NCT01723696) and in 33 pregnant nonsmokers. Doppler ultrasound measurements showed decreased umbilical vein Doppler velocity (Vmax) in placebo-treated smokers that was significantly improved in smokers randomized to vitamin C, restoring to levels comparable to nonsmokers. RNA-sequencing demonstrated that vitamin C supplementation to pregnant smokers was associated with changes in mRNA expression in genes highly relevant to vascular and cardiac development, suggesting a potential mechanism for vitamin C supplementation in pregnant smokers to improve some aspects of offspring health.

Despite strong smoking cessation efforts, over 50% of smokers who become pregnant will continue to smoke during pregnancy[1,2]. Maternal smoking during pregnancy (MSDP) affects the development of the placenta and multiple fetal organ systems including lung, brain, kidney and vasculature[3–6]. Offspring of women who smoked during pregnancy have decreased lung function caused by narrowing and increased branching of airways[7,8]. The vasculature of offspring of smokers is affected by increased intimal and medial thickness and they show modest increases in blood pressure and decreased renal glomerular filtration rates[9–12].

Not surprisingly, MSDP also effects placental structure and function. Both animal studies and histologic analysis of placentas from pregnant human smokers show that nicotine accumulates in amniotic fluid and freely crosses the placental barrier[13] where it can bind to nicotinic acetylcholine receptors expressed in multiple placental cell types[14] to potentially alter placental development. MSDP is associated with increased connective tissue in the placenta and thickening of the trophoblastic basement membrane[15]. Doppler ultrasound (US) measurements in pregnant smokers show increased umbilical artery pulsatility index, suggesting increased placental vascular resistance, and decreased umbilical venous volume blood flow, consistent with reduced blood

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supply to the fetus[3,16,17]. Along with the decrease in placental function there is an increase in relative weight of placentas after correction for the intrauterine growth restriction (IUGR) associated with MSDP, such that the ratio of fetal weight to placental weight (F/P ratio) is decreased in offspring of smokers; suggesting a potential compensatory mechanism for a less robust placental unit that is growing larger to meet the oxygen demands of the fetus[18,19].

In nonhuman primates (NHP), we have demonstrated that the negative effects of nicotine on offspring lung function are primarily caused by nicotine crossing the placenta to interact with nicotinic receptors that are highly expressed in fetal lung[20]. NHP exposed to nicotine in utero show decreases in pulmonary function similar to human infants exposed to tobacco smoke in utero, thus supporting the central role of nicotine[21]. This finding has been replicated in multiple other animal models as well[22,23]. In NHPs exposed to nicotine during pregnancy, we have also demonstrated that prenatal vitamin C supplementation mitigated the nicotine-induced changes in offspring pulmonary function[24,25]. This study led to two human double-blind, randomized controlled trials (RCTs) demonstrating significantly improved pulmonary function and decreased wheeze in the offspring of pregnant smokers randomized to vitamin C supplementation in pregnancy[26,27].

We next examined the impact of prenatal nicotine exposure on placental function in NHP, as well as the potential protective effect of vitamin C supplementation. As we have previously reported[25], prenatal nicotine exposure in NHP resulted in decreased placental blood flow that correlated with placental histological findings and this effect was partially prevented by vitamin C supplementation. Based on our NHP studies, we hypothesized that placental hemodynamics would be similarly improved in human smokers who were given supplemental vitamin C during pregnancy.

To test this hypothesis, we conducted an ancillary study in a subset of participants in the "Vitamin C to Decrease Effects of Smoking in Pregnancy on Infant Lung Function" (VCSIP; NCT#01723696) RCT, using Doppler-US to quantify the effect of vitamin C supplementation on measures of placental hemodynamics. Informed by our prior NHP studies, we chose to assess the umbilical artery pulsatility index (UAPI) as an indicator of vascular impedance, umbilical vein doppler velocity (Vmax) and the calculated quantification of umbilical vein blood volume (cQuv) as a comprehensive measure that accounts for both blood velocity and vessel lumen size. In addition, to better understand the underlying mechanisms for the protective effect of supplemental vitamin C on placental function in MSDP, we also performed placental transcriptome profiling to identify changes in gene expression associated with vitamin C supplementation.

Results

Figure 1 shows the overall design of the study and Table 1 presents the maternal and infant demographic and birth statistics per group (nonsmoker, placebo supplemented-smoker, vitamin C supplemented-smoker) for the 88 subjects who underwent ultrasound assessment. As expected, fasting plasma ascorbic acid (AA) levels were increased in vitamin C-supplemented smokers versus placebo at mid- and late-gestation. Maternal body mass index (BMI) at enrollment was also higher in the placebo group ($p=0.02$). Tobacco exposure as measured by self-report and maternal cotinine were similar between the randomized-smoking groups across pregnancy. The demographics for this subpopulation did not differ significantly from the previously published demographics of the entire study population[28].

Third trimester doppler ultrasound (Doppler-US)

Mean umbilical vein Doppler velocity was significantly decreased in placebo-treated smokers compared to nonsmokers and vitamin C during pregnancy restored levels to that of nonsmokers (Fig. 2A; Table 2). Mean umbilical vein volume blood flow (cQUV) trended downward in placebo-treated smokers compared to nonsmokers (209.4 ± 14.6 vs. 254.9) and was partially restored in smokers randomized to vitamin C compared to nonsmokers (249.2 ± 16.0 vs. 254.9 ± 14.2 mL/min), (Fig. 2B; Table 2). Mean umbilical artery pulsatility index (PI) trended upward in both placebo- supplemented and vitamin C-supplemented smokers compared to nonsmokers and was not decreased by vitamin C supplementation (Fig. 2C; Table 2). There were no significant differences between groups in terms of gestational age at ultrasound, intraabdominal umbilical vein diameter, intraabdominal umbilical vein cross sectional area, or fetal heart rate (Tables 1 and 2).

Placental histology

The standard "Amsterdam criteria"[29] was used to evaluate and score for a comprehensive range of potential placental pathology including chorangiosis (placental villous capillary growth). In general, the placentas from the two randomized-smoking groups had more histologic abnormalities than the placentas from the non-smoking group (Supplementary Table S1). However, the histologic analysis did not show any meaningful differences between the placebo-supplemented and vitamin C-supplemented subjects other than 2 cases of acute chorioamnionitis in the vitamin C-supplemented group (Supplementary Table S1). Differences were not detected between area and intensity of trichrome staining in placental villi or blood vessels between vitamin-C treated subjects and smokers, though the area of trichrome staining surrounding blood vessels was increased in the non-smoker group (data not shown).

While there was only a slight increase in the fetal/placental weight ratio (F/P ratio) in smokers randomized to vitamin-C treatment compared to placebo, there was a significant correlation between offspring pulmonary function at 5 years of age and the F/P ratio in the nonsmoker and vitamin-C treated groups that was absent in the placebo-treated group (Fig. 3).

RNA-seq differential gene expression

Eighty placental samples were selected for RNA-sequencing using a blocked randomization design described in the methods. Seven samples were removed from analysis as outliers based on correlation of PCs with

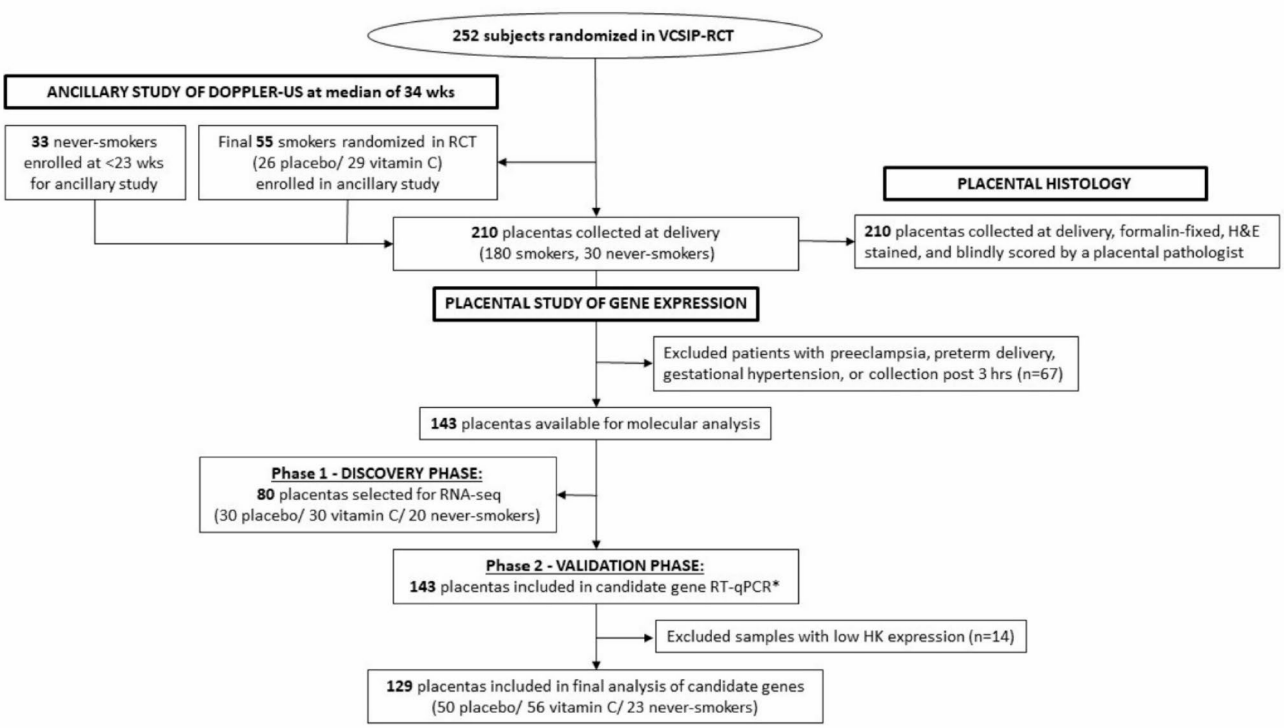

Fig. 1. Consort diagram and study design. Pregnant smokers were randomized to vitamin C or placebo at less than 23 weeks of gestation as part of a clinical trial to evaluate impact on offspring lung function. Non-smoking subjects were recruited as part of an ancillary study to perform doppler ultrasound. Placentas were collected at delivery from 210 subjects for histology and gene expression analysis. Samples with pregnancy-related complications and poor-quality control metrics were excluded from molecular analysis.

seqQC metrics, suggesting technical problems with the libraries (3 placebo, 2 vitamin C, and 2 nonsmokers). The primary analysis compared placental expression in pregnant smokers randomized to placebo ($n=27$) versus vitamin C-supplemented pregnant smokers ($n=28$), adjusted for batch, infant sex, and cell type PC1 (Supplemental Materials). Out of 19,927 genes included in analysis, 613 were nominally significant between randomization groups (Fig. 4A; $p<0.05$), of which 313 were up-regulated and 300 down-regulated in the placebo-smokers versus the vitamin C-smokers. Two genes reached FDR significance in the comparison of placebo supplemented-smokers to nonsmokers: *CYP1A1* and *TGFB1* (both upregulated), and 2222 genes were nominally significant (Fig. 4B; $p<0.05$; 838 up-regulated; 1384 down-regulated in placebo group). The overlap of nominally significant genes ($n=164$) between the two comparisons (placebo vs. vitamin C-supplemented smokers; placebo-supplemented smokers vs. nonsmokers) is illustrated in Fig. 4C. Out of 164 genes overlapping between the two comparisons, 160 were normalized with vitamin C supplementation by at least 40% toward the level of nonsmokers (Fig. 4D). The complete table of differential expression is shown in Supplementary Table S2.

Functional and pathway enrichment analyses

ConsensusPathDB analysis of the 164 normalized genes identified "circulatory system development ", "blood vessel morphogenesis", "vascular development", "cardiovascular system development", and "cellular response to toxic substance" as the top 5 Gene Ontology (GO) terms (Table 3). Qiagen Ingenuity Pathway Analysis of all nominally significant DEGs between placebo and vitamin C-supplemented groups, predicted "Nerve growth factor (NGF)-stimulated transcription" as the top canonical pathway and we list all 52 canonical pathways with a significant activation score in Supplementary Table S3. A total of 2050 upstream regulators were predicted (Supplementary Table S4), including 99 targets downstream of lipopolysaccharide.

Fluidigm RT-qPCR validation of candidate genes

All placentas passing quality control metrics from the parent RCT and from consented nonsmokers (Fig. 1) were included in the validation of candidate genes by RT-qPCR. The characteristics of this subset ($n=129$) are provided in Supplementary Table S7, and the primers used for the Fluidigm amplification in Supplementary Table S8. Out of 20 candidate genes measured by RT-qPCR, 8 were validated as differentially expressed (nominal $p<0.05$ and consistent direction of effect) between randomized treatment groups (Supplementary Tables S5, S6). Figure 5 shows expression boxplots for 3 candidate genes (*JUN*, *CCN1*, and *EGR1*), as well as the interaction network of validated genes.

	Nonsmokers (n=33)	Placebo-treated smokers (n=26)	Vitamin C-treated smokers (n=29)	p-value vitamin C vs. placebo
Maternal characteristics at randomization				
Age at enrollment, median (IQR), years	32 (29–33)	24 (22.25–32)	28 (21–32)	0.953
Race, n white (%)	30 (90.9)	21 (80.8)	27 (93.1)	0.308
Gravida, median (IQR)	2 (1–3)	2.5 (1–4.8)	3 (1–3)	0.753
Body mass index at enrollment, mean (SE) kg/m^2	27.83 (1.01)	30.78 (1.37)	26.71 (0.79)	**0.020**
History of asthma, n (%)	5 (15.2)	5 (19.2)	10 (34.5)	0.335
Private health insurance, n (%)	31 (93.9)	5 (19.2)	4 (13.8)	0.471
Gestational age at randomization, median (IQR), weeks	NA	17.75 (14.8–19.53)	17.9 (14.7–19.4)	0.627
Maternal cigarettes/day during pregnancy, median (IQR)	NA	8 (4.25–10)	8 (5–10)	0.898
Urine cotinine at randomization, median (IQR), ng/mL	Not detectable	5197 (1885–8302)	5418 (1872–7278)	0.959
Plasma ascorbic acid at randomization, mean (SE), µmol/L	Not measured	41.9 (3.1)	46.2 (3.3)	0.416
Plasma ascorbic acid at mid-gestation mean (SE), µmol/L	Not measured	32.2 (2.8)	57.9 (4.3)	**<0.001**
Plasma ascorbic acid at late-gestation mean (SE), µmol/L	Not measured	33.47 (3.1)	45.7 (3.9)	**0.016**
Patient characteristics at Doppler ultrasound				
Time from last cigarette, median (IQR), h	NA	1.3 (0.58–1.41)	1.11 (0.53–1.46)	0.454
Gestational age at ultrasound, median (IQR), weeks	35 (33.4–36.2)	32.8 (32.3–34.3)	34.2 (32.3–35.2)	0.423
Birth characteristics of infants				
Birth weight, mean (SE), g	3571.8 (76.3)	3175.9 (97.1)	3142.4 (105.2)	0.958
Placental weight, mean (SE), g	Not measured	584.45 (142.75)	612.19 (119.88)	0.551
Gestational age at birth, median (IQR), weeks	39.6 (39.1-40.0)	39.1 (38.3-39.6)	38.6 (37.3-39.6)	0.140
Female, n (%)	19 (57.6)	12 (46.2)	11 (37.9)	0.731
Preterm, <37 weeks gestation, n (%)	0 (0)	0 (0)	5 (17.23)	0.080

Table 1. Population characteristics of mothers and infants with completed doppler ultrasound. Significant values are in bold. Normally distributed variables are expressed as mean and standard error and compared for group differences using an unadjusted F-test. Non-normally distributed variables are expressed as median and interquartile range (25th–75th percentile) and the Wilcoxon rank sum test was used to compare groups. Chi-square test was used to compare categorical variables.

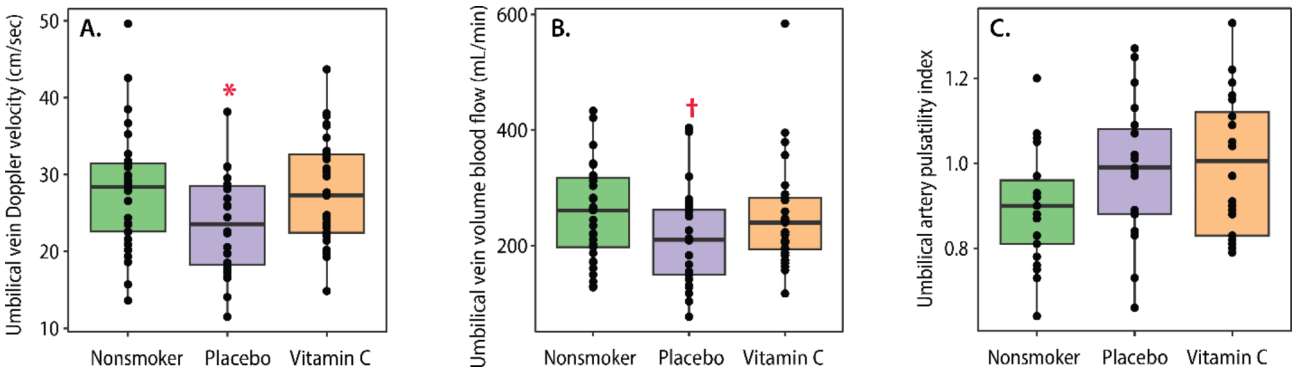

Fig. 2. Comparison of Doppler ultrasound measures. Boxplots show effects by treatment group on (**A**) Umbilical vein Doppler velocity; (**B**) Umbilical vein volume blood flow; (**C**) Umbilical artery pulsatility index. Plots show median and interquartile ranges. * $p < 0.05$ compared to nonsmokers and vitamin-C treated groups, † $p < 0.09$ compared to vitamin-C treated group by F-test using mixed linear model per measurements adjusted for gestational age at ultrasound.

Discussion

This study demonstrates that supplementation with vitamin C (500 mg/day) to pregnant smokers results in umbilical vein Doppler blood flows comparable to nonsmokers. Multiple studies have established that MSDP, as well as prenatal nicotine exposure alone, are associated with decreased placental vascular function as assessed by Doppler-US and abnormal placental histology[3,16,30]. Our results in this clinical population are consistent with our prior observation in NHPs that vitamin C supplementation mitigated nicotine-induced detrimental changes in placental hemodynamics and correlated with improved offspring lung function[24,25]. In other studies using

	Nonsmokers (n=33)	Placebo-treated smokers (n=26)	Vitamin C-treated smokers (n=29)	p-value nonsmoker vs. placebo	p-value nonsmoker vs. vitaminC	p-value vitamin C vs. placebo
Umbilical vein Doppler velocity (Vmax), cm/s	28.00 ± 1.31	23.49 ± 1.10	27.88 ± 1.21	0.05	0.92	0.04
Umbilical vein volume blood flow (cQUV), mL/min	254.9 ± 14.2	209.4 ± 14.6	249.2 ± 16.0	0.17	0.71	0.09
Umbilical artery pulsatility index (UAPI)	0.90 ± 0.02	0.99 ± 0.03	0.99 ± 0.03	0.07	0.05	0.95
IAUV—intraabdominal umbilical vein diameter (IAUV), cm	0.62 ± 0.02	0.61 ± 0.02	0.61 ± 0.01	0.85	0.78	0.93
Cross sectional area of the umbilical vein (CSA), cm^2	0.31 ± 0.02	0.30 ± 0.02	0.30 ± 0.01	0.83	0.75	0.94
Fetal heart rate (FHR), beats/min	143.0 ± 1.6	137.3 ± 1.9	141.3 ± 2.0	0.02	0.59	0.06

Table 2. Doppler ultrasound measurements of fetal and placental hemodynamics. Data shown as mean ± SE. CSA calculated as π(UV diameter (cm)/2)^2; cQUV calculated as 0.5 × Vmax × CSA × 60. P-values for F-test using mixed linear model per measurement adjusted for gestational age at ultrasound and BMI. Logarithmic transformation were performed for the following measurements to reduce skewness of residuals: IAUV, CSA, cQUV, and FHR.

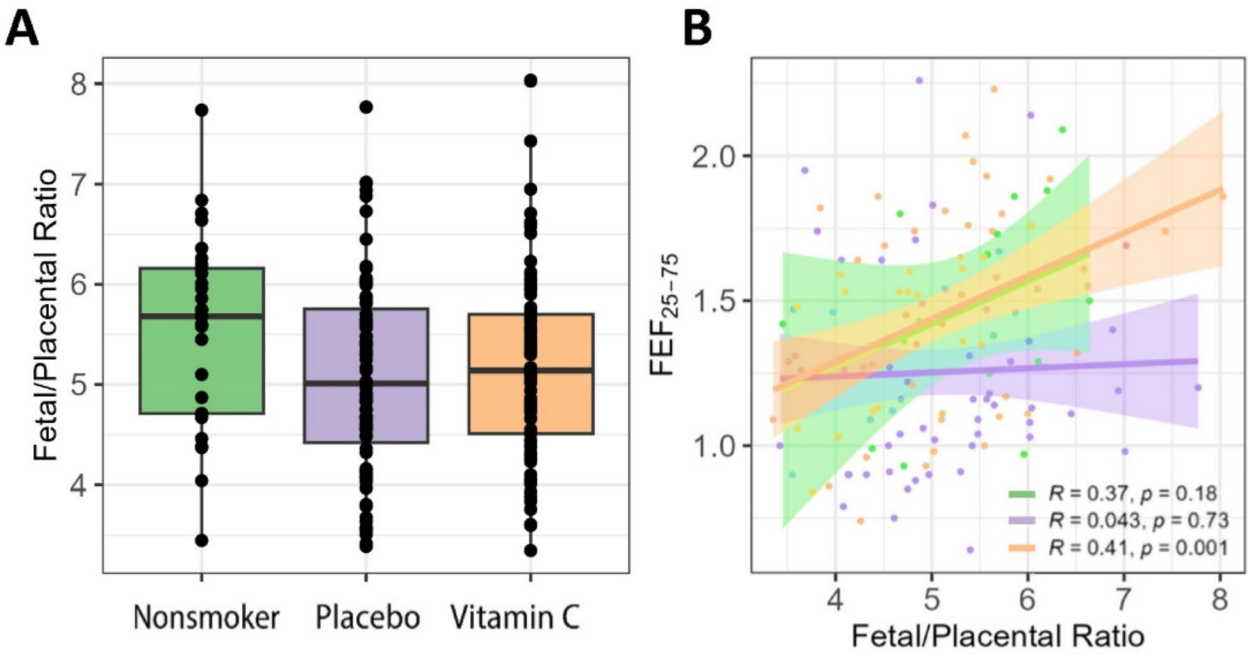

Fig. 3. Fetal/placental weight ratio by group and correlation with pulmonary function at 5 years of age. (**A**) Boxplot shows the distribution by treatment group. (**B**) Scatterplot shows the Pearson correlation between fetal/placental weight ratio and FEF_{25-75} measured at 5-year follow-up visit for each treatment group. The correlation was significant in the vitamin C treated smokers (orange) with a similar trend in nonsmokers (green), however this relationship was not observed in placebo treated smokers (purple).

animal models, supplemental vitamin C appears to increase the velocity of airway flows by preventing nicotine-induced alterations in airway geometry[22,24,31]; thus, vitamin C may act similarly in the placenta by preventing alterations in vascular geometry.

Histological studies on placentas of smokers have reported increased thickness of the villous membrane, increased collagen deposition, decreased vascularization of the placental bed, as well as reduced intervillous area and capillary volume[15]. These changes in the placenta are similar to changes observed in the aorta and other blood vessels in offspring of smokers[30,32], suggesting that the mechanism by which vitamin C improves placental blood flow in smokers may resemble the widespread effects of MSDP on pulmonary and cardiovascular development. While we did observe more histologic abnormalities in the placentas from the two randomized groups of smokers, there was little difference observed between the placebo and vitamin C-supplemented groups. Only minor differences in trichrome staining intensity was seen in the placentas from nonsmokers compared to smokers, perhaps representing our limited sample size, or the fact that average smoking intensity

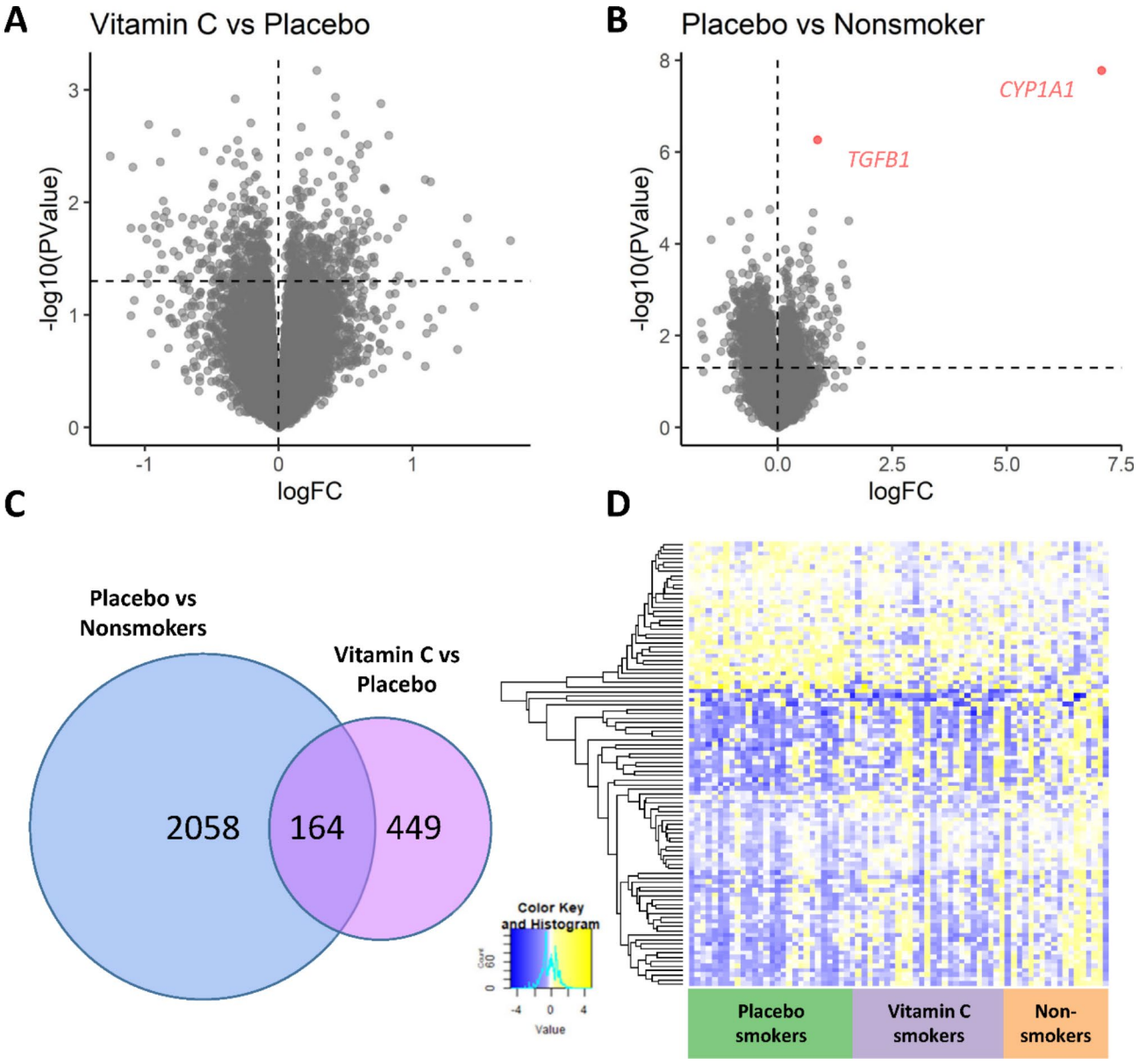

Fig. 4. RNA-sequencing results of placentas. Volcano plots show difference in log fold change (log FC) versus –log10 p-value in vitamin C vs. placebo (**A**) and placebo vs. nonsmokers (**B**) Lines at y = 1.3 are equivalent to nominal $p = 0.05$. (**C**) Venn-diagram shows overlap of nominally significant changes in expression between groups. (**D**) Heatmap for overlapping set of 164 genes shown in **C** with nominally significant differential expression in placentas from smokers randomized to placebo vs. nonsmokers and in placentas from placebo vs. vitamin C-treated smokers. Columns represent individual subjects and rows represent individual genes. The mean direction of effect in 160/164 overlapping genes was reversed with vitamin C.

has decreased over time[33,34]. Because of the small differences between the two randomized groups of smokers, further stereometric analysis was not indicated for this study.

Consistent with the findings of decreased vascularization on placental histology and increased umbilical artery PI in both smoking groups combined, by Doppler ultrasound the fetal-side placental blood flow (cQUV) was lower in smokers compared to nonsmokers and smokers randomized to vitamin C supplementation. It is important to note however that we did not see a significant difference in birth weight wit vitamin C supplantation which suggests that while smoking may reduce placental blood flow and oxygen delivery to the fetus, there are likely other contributing factors affecting infant birth weight.

A prospective study of Doppler waveforms at term, reported increased pulsatility index (PI) and resistance in the umbilical artery measurements of pregnant smokers that negatively correlated with infant birth weight[35]. In this study, we saw no difference in umbilical artery PI between randomized treatment groups. However, umbilical artery PI was increased in both smoking groups (vitamin C-supplemented smokers and placebo-supplemented smokers) combined versus nonsmokers and was negatively correlated with infant birthweight

Gene Ontology (GO) description	q-value
Circulatory system development	0.002
Blood vessel morphogenesis	0.002
Vasculature development	0.002
Cardiovascular system development	0.002
Cellular response to toxic substance	0.003
MAPK cascade	0.008
Atrial septum development	0.012
Drug transport	0.012
Negative regulation of cellular process	0.012
Negative regulation of signaling	0.014

Table 3. Top 10 enriched GO terms from ConsensusPathDB. Input to analysis included 164 genes nominally significant in comparison of placebo vs. never-smokers and placebo vs. vitamin C; q-value = p-value corrected for multiple testing using the false discovery rate method.

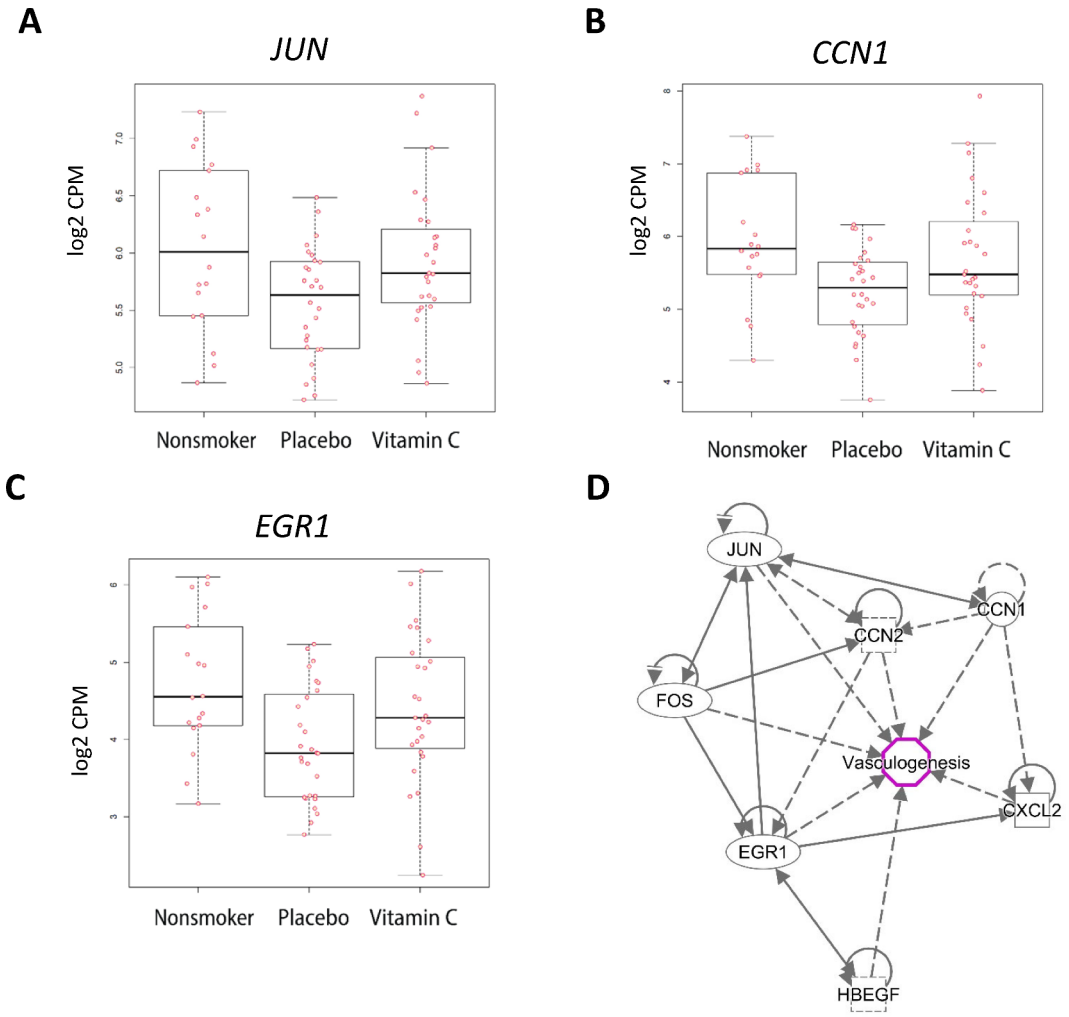

Fig. 5. Top differentially expressed genes validated by qPCR. (**A**–**C**) Boxplots for top 3 genes with significant differential expression in placentas from smokers randomized to placebo vs. nonsmokers and in placentas from placebo- or vitamin C-treated smokers validated by qPCR. (**D**) IPA analysis of nominally significant overlap identified enrichment of canonical signaling related to vasculogenesis. All genes shown were significantly upregulated in vitamin C supplemented smokers versus placebo and in never smokers vs. placebo.

(data not shown). This suggests that the protective effects of vitamin C on placental blood flow may be acting independently of the effects of smoking on the umbilical artery PI. As the umbilical artery PI is clinically used as a measure of placental vascular resistance, this greater effect on placental blood flow than umbilical artery PI mirrors the effects of vitamin C supplementation on lung development and function in which vitamin C significantly increases forced airway expiratory flows, but has less effect on airway resistance[11,24].

There is also an interesting potential interaction between vitamin C and fetal-placental weight ratio (F/P) on offspring airway function (Fig. 3). Multiple studies have demonstrated a decreased F/P weight ratio in smokers[18,19]. While the underlying etiology for the observed increased placental weight associated with smoking is unclear, it may represent an adaptive placental response to the decreased placental functionality. Thus, in placebo-supplemented smokers a larger placenta does not correlate with improved placental function, and we saw no correlation of the F/P weight ratio with offspring pulmonary function in the placebo-supplemented smokers. However, in the vitamin C-supplemented smokers and nonsmokers, there was a clear correlation between F/P ratio and offspring pulmonary function (Fig. 3) suggesting that vitamin C might be restoring an optimal balance between placental function and placental weight. This is consistent with the well-established link between lung development and placental development[36,37], though the finding reported here needs further investigation.

Placental expression analysis by RNA-sequencing suggests normalized expression of genes involved in vasculogenesis, endothelial tissue development, and response to growth factors as potential mechanisms. The expression of genes critical to these processes was confirmed by RT-qPCR. Of note, vitamin C supplementation was associated with decreased expression of *SLC23A2* (solute carrier family 23 member 2), which encodes one of two vitamin C transporter proteins and is important for ascorbic acid transport across the placenta[38]. We also observed normalized expression of two members of the CCN family of secreted matricellular proteins, named for the first family members discovered: *CYR61* (cysteine-rich protein 61), *CTGF* (connective tissue growth factor), and *NOV* (nephroblastoma overexpressed gene). *CYR61* and *CTGF* are immediate early genes, a class of genes that are rapidly inducible in response to growth factors[39]. *CYR61* induces angiogenesis in fibroblasts and endothelial cells through interaction with integrin receptors[40] and *Cyr61*-null mice exhibit placental vascular insufficiency and compromised vessel integrity[41]. In our study, vitamin C also normalized mean expression of *FOS* (Fos proto-oncogene) and *JUN* (Jun proto-oncogene), to the mean level of nonsmokers. In agreement with our results, vitamin C increased mRNA expression of *c-jun* and *c-fos* in HL-60 cells induced to differentiation by 1α,25(OH)_dihydroxyvitamin D_3[42]. Vitamin C also normalized levels of heparin binding EGF like growth factor (*HBEGF*) and HB-EGF has been reported to play a role in preventing oxidative stress-mediated uterine decidual damage[43].

Comparison of smokers to nonsmokers revealed an increase in placental cytochrome P450 (CYP) enzyme (*CYP1A1)* expression that was not improved with vitamin C supplementation. Notably, elevated *CYP1A1* activity in placentas of smokers has been associated with several adverse health outcomes including preterm delivery, intrauterine growth restriction, and lower infant birth weight[44]. Placental induction of *CYP1A1* in response to cigarette smoke is epigenetically regulated and hypomethylation of the *CYP1A1* promoter may serve as an epigenetic record of exposure to maternal smoke that is irreversible[45–47]. We also observed a small but genome-wide significant increase in transforming growth factor-beta (*TGFB1*) in smokers versus nonsmokers, in contrast to what has been reported previously[48]. TGF-β regulates proliferation and differentiation of placental trophoblast cells, in addition to regulating placental steroidogenesis[49]. The role of TGF-β signaling in vascular development is complex and both pro-angiogenic and anti-angiogenic properties have been described[50]. Our previous study of placental DNA methylation in this RCT cohort also identified epigenetic dysregulation of *TGFB1* and *CYP1A1* expression[51].

Vitamin C has been shown to reduce blood pressure, especially in patients with hypertension and low plasma levels of vitamin C at baseline. The proposed mechanisms for vitamin C to decrease hypertension include improvement of arterial compliance, redox status and endothelial dysfunction, increased nitric oxide and prostaglandin I2, and reduced leukotrienes and lipids[52]. In our study, nitric oxide synthase 3 (*NOS3*) was significantly increased in placentas randomized to placebo-supplementation versus nonsmokers but was not restored by vitamin C supplementation in our wider qPCR analysis. These results agree with our observation that vitamin C supplementation did not normalize umbilical artery PI.

Our study had three major strengths. First, our study population was nested within a double blind, placebo-controlled RCT of vitamin C supplementation (500 mg/day) to pregnant smokers with extensive phenotypic and clinical data that demonstrated significantly increased airway function through 5 years of age. Second, our study used whole-genome transcriptome profiling with RNA-seq to identify changes in placental gene expression, which allows for unbiased discovery of enriched molecular pathways and has not been previously performed. Third, we validated a subset of genes relevant to vascular biology from the discovery phase using qPCR in the larger RCT population.

The major limitation to our study was the relatively small sample size of subjects who underwent Doppler ultrasounds as part of this ancillary clinical study. However, we were still able to demonstrate a significant increase in umbilical vein Doppler velocity in addition to other altered Doppler-US values. Given our sample size, we did not exclude patients from undergoing Doppler-US because of pregnancy complications and ultimately 17% ($n=5$) of the vitamin C versus none of the placebo-supplemented smokers delivered preterm, although removing patients who delivered preterm would have further increased the vitamin C effect size on cQUV. Another limitation of the study is that maternal side placental blood flow was not included due to the challenge of achieving rigorous and reproducible doppler measurements in the third trimester, which would have provided additional insight into placental function. We would however expect to have similar findings to a prior study where we performed both maternal and fetal placental doppler measurements to examine the impact

of prenatal alcohol[53]. In that study, we observed that uterine artery doppler blood flow followed a similar trend as the umbilical artery doppler blood flow measurements between alcohol-exposed and control placentas.

In conclusion, our study demonstrates that supplementation of vitamin C to pregnant smokers has the potential to improve placental hemodynamics. The umbilical venous flow directly contributes to the amount of oxygen and nutrients that reach the fetus[54], suggesting that vitamin C supplementation to pregnant smokers may lessen the deleterious actions of MSDP by increasing blood supply to the fetus. The potential mechanisms for the observed effects of vitamin C in increasing placental volume blood flow in parallel with changes in offspring pulmonary function may include amelioration of altered vascular geometry associated with nicotine exposure. Pathway analysis of RNA-sequencing results suggest activation of growth factor signaling cascades, blood vessel morphogenesis, and cardiovascular system development. These findings are in line with our previous study of restored placental DNA methylation in this RCT at loci associated with infant lung function[51]. Future pragmatic clinical studies are needed to confirm these findings in actual obstetrical practice to determine the extent to which vitamin C supplementation for pregnant smokers should be included in standard practice[55].

While the clinical impact of the observed placental function changes on fetal development and obstetric outcome is unknown, this finding, combined with the transcriptomic results, suggests that perturbations to fetal hemodynamics by MSDP may be ameliorated by vitamin C supplementation. This supports the findings of our prior studies highlighting the benefits of vitamin C supplementation to mitigate some aspects of offspring morbidity, particularly respiratory morbidity, in pregnancies where abstaining from nicotine products is not feasible. It is important to note however that there is no data to suggest that vitamin C supplementation will affect the majority of effects of smoking on fetal development such as prematurity, neural development, and overall somatic growth.

Materials and methods

Study design

This study was nested within a multi-center, double-blind RCT which demonstrated improved airway function at 3, 12 and 60 months of age in offspring whose mothers were randomized to vitamin C (500 mg/day) versus placebo[27,28,56]. Recruitment of participants into the parent study began in December 2012 and ended in December 2015. All subjects randomized after August 2014 were consented into an ancillary study to undergo Doppler-US. A group of 33 pregnant nonsmokers were enrolled as a reference group and followed prospectively according to a modified protocol similar to the randomized pregnant smokers (Fig. 1). The study of gene expression was divided into 2 phases: (1) the discovery phase that included total RNA-sequencing of 80 stratified, randomly sampled placental samples and (2) the validation phase of expression of 20 candidate genes by RT-qPCR from all available placental samples meeting inclusion criteria.

Study population

The parent RCT recruited women with singleton pregnancies ($\geq$15 years old; between $13^0/_7$ and $<$23 weeks gestation at randomization) with a history of current smoking and documented refusal/inability to quit. Inclusion criteria included current cigarette smoking of at least 1 cigarette in the last week, collected at randomization. Women were randomized to receive supplemental vitamin C versus placebo in rotations of two and four subjects, and stratified by gestational age at randomization ($\leq$18 versus $>$18 weeks) and site (Oregon Health & Science University [OHSU], Portland, Oregon; PeaceHealth Southwest Medical Center [SWW], Vancouver, Washington; Indiana University [IU], Indianapolis, Indiana). Both randomized groups received standard prenatal vitamin with minimum daily requirements of vitamin C. A total of 252 pregnant smokers were randomized and 243 infants were available for study at delivery.

The RCT was approved by each site's Institutional Review Board (OHSU Institutional Review Board and Indiana University Institutional Review Board) and the study itself was monitored by an NIH appointed Data Safety Monitoring Board (Vitamins for Early Lung Health (VITEL)). We obtained written informed consent from all subjects prior to enrollment[57]. All methods and procedures were performed in accordance with the relevant guidelines and regulations.

Third trimester doppler ultrasound (Doppler-US)

At a median of 34 weeks of gestation, a sonographer blinded to the treatment group performed Doppler-US using image-directed pulsed and color Doppler equipment (Phillips & GE Voluson). The lowest high-pass filter was used and an angle of 30 degrees or less between the Doppler beam was deemed acceptable[58]. Doppler waveform measurements in 55 randomized smokers and in 33 pregnant nonsmokers were obtained from the straight portion of the intraabdominal umbilical vein (IAUV) as described previously[25,59,60]. The cross-sectional area (CSA) of the IAUV was calculated as $CSA = \pi\ (\text{diameter}/2)^2$, and Vmean was calculated as 0.5 $\times$ Vmax. The umbilical vein volume blood flow was calculated as Vmean $\times$ CSA $\times$ 60[54,61]. The umbilical artery waveform was measured in a free-floating loop of the mid-umbilical cord using machine-specific software to obtain the pulsatility index (PI) and the fetal heart rate[62]. An obstetrician and a Maternal-Fetal Medicine subspecialist (A.E.F.) blinded to treatment group reviewed all ultrasound images. For each measurement, three consecutive uniform wave-forms were recorded and the mean of three measurements was used for further analysis.

Placental samples

Following delivery, placentas were collected and processed within 3 h of delivery by trained research staff using a standardized protocol[57]. Fresh placentas were weighed after trimming off the cord and measured (length, width, and average thickness), positioned fetal side up, and fetal membranes folded around the edge. Four quadrants were mapped out visually and cores collected from each quadrant in a radial pattern within 4 cm of the cord insertion site using a 1 cm punch, avoiding major vessels. Each core was rinsed in saline to remove

maternal blood, trimmed of maternal decidua and fetal membranes, and cut lengthwise to yield 8 villous samples total. Four samples were formalin-fixed for histological analyses (one per quadrant), 2 were collected into RNAlater to provide RNA/DNA, and 2 were flash frozen. For molecular analyses one RNAlater treated sample per subject was submerged in liquid nitrogen in a pre-chilled mortar and pestle and ground into a fine powder. We simultaneously extracted RNA, miRNA, and DNA from placental powder using the Qiagen AllPrep Universal Kit and the QIAcube for automated nucleic acid extraction (Qiagen, USA). Fixed placental tissue was hematoxylin and eosin (H&E), and Mallory trichrome-stained, and scored by a board-certified placental pathologist (T.K.M.), blinded to treatment group, according to Amsterdam criteria[29]. All placental samples were reviewed three times to ensure that the placental findings were reproducible.

Cell type deconvolution

Bulk RNA-seq represents a convolution of gene expression from a mixture of cell types. We therefore used a publicly available cell type deconvolution reference signature matrix, developed using single-cell sequencing of healthy term placental samples, to estimate proportions of 19 fetal cell types and 8 maternal cells[63]. The signature matrix and our mixture file were input to CIBERSORTx with default settings and 1000 permutations to evaluate imputation goodness-of-fit. We then performed principal component analysis of estimated cell proportions, and determined that principal component 1 (PC1) represented 98.39% of variance[63].

RNA-seq differential expression analysis

For molecular analyses, we excluded subjects with gestational hypertension, preeclampsia, and preterm delivery (< 37 weeks), and placentas sampled greater than 3 h after delivery (Fig. 1). We used a blocked randomization design to randomly select initial placental RNA samples for sequencing with 10 blocks of 8 (3 placebo, 3 vitamin C, 2 nonsmokers). We constructed a model representing the study design (~ group + batch + sex + cell PC1) and used glmQLFit[64] to fit the negative binomial generalized linear model for each gene. Genes with nominally significant differential expression were included in functional and pathway enrichment analyses using ConsensusPathDB[65] and Ingenuity Pathway Analysis (Qiagen Inc., MD, USA)[66].

Fluidigm biomark gene expression by RT-qPCR

We performed candidate gene validation of RNA-seq results by RT-qPCR using the Fluidigm Biomark system and 96.96 Dynamic Array IFCs (Fluidigm, CA, USA) according to the Fluidigm Delta Gene Assay protocol. We calculated delta Ct (ΔCt) for each sample and assay (target): ΔCt = mean Ct of target − mean Ct of housekeeping genes (combined mean Ct from *RPL19*, *TOP1*).

Statistical analysis

For comparison of Doppler measurements between groups, we followed a pre-defined statistical design to give 80% power at level 0.05 to detect a difference of 0.12 in umbilical artery PI between pairs of groups. To achieve this we projected to enroll 33 subjects for each of the three groups (nonsmokers, smokers randomized to placebo, and smokers randomized to vitamin C). Assuming a drop-out rate of 2.4% from randomization until the 34 week ultrasound, this would yield 32 subjects per group and give 80% power at level 0.05 to detect a difference of 0.12 in umbilical artery PI between pairs of groups based on SD's of 0.09 and 0.22[67] We constructed linear regression models for each measurement adjusted for gestational age at ultrasound (GAUS) and maternal BMI as continuous values. Normality was assessed for each measurement using the Shapiro–Wilks test and logarithmic transformations performed as appropriate.

For demographic variables presented in Table 1, normally distributed variables were expressed as mean and standard error and compared for group differences using an unadjusted F-test. Non-normally distributed variables were expressed as median and interquartile range (25th–75th percentile), and the Wilcoxon rank sum test was used to compare groups. Chi-square test was used to compare categorical variables. Significance was defined as $p < 0.05$. We performed all statistical analyses using "*EdgeR*," "*stats*", "*car*", and "*emmeans*" packages in R version 3.6.1. Due to our limited sample size, we did not adjust for multiple testing and considered a nominal $p < 0.05$ as significant.

Data availability

The raw and processed RNAseq data is publicly accessible through the NCBI Gene Expression Omnibus (GEO) via accession series GSE253158.

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Author contributions

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Declarations

Competing interests

The authors declare no competing interests.

Additional information

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