



Exposure to fine particulate matter 2.5 from wood combustion smoke causes vascular changes in placenta and reduce fetal size



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ABSTRACT

During gestation, maternal blood flow to the umbilical cord and placenta increases, facilitating efficient nutrient absorption, waste elimination, and effective gas exchange for the developing fetus. However, the effects of exposure to wood smoke during this period on these processes are unknown. We hypothesize that exposure to PM2.5, primarily sourced from wood combustion for home heating, affects placental vascular morphophysiology and fetal size. We used exposure chambers that received either filtered or unfiltered air. Female rats were exposed to PM2.5 during pre-gestational and/or gestational stages. Twenty-one days post-fertilization, placentas were collected via cesarean section. In these placentas, oxygen diffusion capacity was measured, and the expression of angiogenic factors was analyzed using qPCR and immunohistochemistry. In groups exposed to PM2.5 during pre-gestational and/or gestational stages, a decrease in fetal weight, crown-rump length, theoretical and specific diffusion capacity, and an increase in HIF-1 α expression were observed. In groups exposed exclusively to PM2.5 during the pre-gestational stage, there was an increase in the expression of placental genes Flt-1, Kdr, and PIGF. Additionally, in the placental labyrinth region, the expression of angiogenic factors was elevated. Changes in angiogenesis and angiogenic factors reflect adaptations to hypoxia, impacting fetal growth and oxygen supply. In conclusion, this study demonstrates that exposure to PM2.5, emitted from wood smoke, in both pre-gestational and gestational stages, affects fetal development and placental health. This underscores the importance of addressing air pollution in areas with high levels of wood smoke, which poses a significant health risk to pregnant women and their fetuses.

1. Introduction

Air pollution has adverse health effects and has become one of the primary public health issues globally. According to the World Health Organization [73], it is estimated that 90% of the world's population lives in areas where the concentrations of fine particulate matter 2.5 (PM2.5) in the air exceed the annual limit deemed safe for health, that is, 5 $\mu\text{g}/\text{m}^3$. Pollution by PM2.5 is associated with 4.2 million premature deaths annually and is responsible for up to 2 million deaths yearly from cardiovascular diseases [21]. Due to their minuscule size, these particles

can carry toxic substances, posing a critical threat to human health [39, 51]. One of the primary emission sources is petroleum combustion, which has been extensively studied. However, emissions from the burning of firewood for indoor heating have been under-researched (Karagulian et al., 2015).

During pregnancy, maternal exposure to PM2.5, primarily sourced from petroleum combustion, has been linked to adverse reproductive outcomes such as low birth weight, intrauterine growth restriction, and prematurity [12,42,55]. These particles can enter the maternal bloodstream, surpass the fetoplacental barrier, and induce endocrine

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disruption and placental inflammation related to oxidative stress and alterations in oxygen transport [5,77]. This inflammation and oxidative stress are associated with the harmful effects of polluted air and disruption of nutrient exchange in the womb, potentially predisposing the fetus to develop cardiovascular diseases and diabetes in adulthood [52]. Consequently, PM2.5 poses a significant risk to pregnant women and their fetuses [15].

Angiogenesis is essential for placental development, supporting the growth and remodeling of the vascular network necessary for efficient oxygen and nutrient exchange between mother and fetus [47]. The invasion and expansion of trophoblastic cells are critical steps in establishing this intricate vascular system within the placenta. These cells differentiate and invade the uterine lining, forming the structural basis of the placenta and initiating the development of the labyrinth zone, where maternal and fetal blood exchange occurs [23]. The principal structures of the placenta include the syncytiotrophoblast, the cytotrophoblast, and the underlying vascular network, all of which are dynamically regulated throughout the various gestation periods [26, 32]. During this time, the placenta undergoes constant architectural and functional changes to meet the increasing demands of the developing embryo. The signaling pathways regulating placental angiogenesis are complex and involve a multitude of factors and growth receptors [2,26, 41]. Vascular endothelial growth factor (VEGF) and its receptors (VEGFR-1/Flt-1, VEGFR-2/Kdr) play a pivotal role in this process, stimulating the proliferation and migration of endothelial cells to form new blood vessels. Placental growth factor (PIGF) acts in conjunction with VEGF and contributes to the angiogenic remodeling that ensures a solid blood supply to the fetus [26,53]. Disruptions in these angiogenic pathways can lead to placental insufficiency and fetal growth restrictions, underscoring the importance of a well-regulated angiogenic environment for successful pregnancy outcomes [25,26,53]. Therefore, understanding the factors and mechanisms of placental angiogenesis not only provides insight into normal fetal development but also has implications for the diagnosis and treatment of pregnancy-related complications.

During the early stage of gestation, hypoxia is the primary trigger for placental angiogenesis [61], with the embryo developing in a low-oxygen environment. In the later stages, an increase in oxygen concentration is essential for proper fetal growth [22]. The transition to an environment with higher oxygen concentration involves challenges and adaptations by the placental tissues, including an increase in their antioxidant defenses to handle the potentially induced oxidative stress by higher oxygen concentrations [6]. This complex balance between hypoxia and adequate oxygenation is essential for healthy placental and fetal development. Hypoxia-inducible factor (HIF-1 α) is a transcription factor that responds to changes in oxygen tension and placental hypoxia, influencing placental development [16]. Chronic hypoxia can affect fetal nutrition and growth due to placental hypoperfusion, leading to fetal growth restriction and preeclampsia [27,29,74].

Temuco - Padre las Casas is a conurbation with historically high levels of PM2.5 suspended in the air, exceeding health-compatible limits, representing an area of intense wood smoke pollution affecting the central-southern and southern regions of Chile, with daily PM2.5 concentrations surpassing 120 $\mu\text{g}/\text{m}^3$ during winter [3,71]. In the year 2021, a peak of 157 $\mu\text{g}/\text{m}^3$ was recorded, and 12 events were reported where concentrations exceeded 100 $\mu\text{g}/\text{m}^3$. The annual average concentration was 26.8 $\mu\text{g}/\text{m}^3$ (± 30.6 ; CV: 117%), five times higher than the limit recommended by the WHO (5 $\mu\text{g}/\text{m}^3$; WHO, 2021). These elevated levels of fine particles in the air are not only alarming due to their environmental implications but also because of their direct impact on public health. Previous research has linked the high presence of PM2.5 in Temuco to an increase in mortality, as well as in rates of hospitalizations and respiratory infections, including cardiovascular and respiratory conditions [57].

In prior studies examining exposure to PM2.5, sourced from wood combustion smoke for indoor heating, we explored the relationship

between air pollution and found that maternal exposure during pregnancy correlates with reduced fetal size and weight [55]. In this study, we assess the impact of pregnant dams' exposure to PM2.5 from wood smoke on fetal development and placental health, comparing exposed and non-exposed groups to discern the differences. We also evaluated the oxygen diffusion capacity and the expression of angiogenic factors to confirm the adaptations to hypoxia. Complementing previous reports, we demonstrate that exposure to PM2.5 from wood smoke significantly affects both the fetus and placental health.

2. Materials and methods

2.1. Exposure site

Sprague-Dawley rats were exposed in Temuco, southern Chile (38°44'59.4"S 72°37'07.8"W), a city identified as the sixth most polluted in Chile [28]. Historically, residential wood-burning stoves have been the primary source of air pollution in this area [13,31,4,57]. During the study, carried out in the southern hemisphere winter season from June 15 to September 30, 2021, the rats were kept under controlled conditions of 20–25°C, with a 12/12-hour light/dark cycle, and had ad libitum access to water and food. No other industrial pollution sources were detected in the area.

2.2. Exposure conditions

We constructed two adjacent exposure chambers in the courtyard of the Faculty of Medicine at the University of La Frontera, located in the downtown area of Temuco, 500 m away from the environmental air monitoring station (supplement 1). This location is known for its historically high levels of air pollution. These chambers, measuring 2.1 m x 2.0 m x 2.1 m, received air at a rate of 20 $\text{m}^3 \text{ min}^{-1}$ and could accommodate up to 50 animal cages. A fan (flow rate of 150 m^3/h , 16.9 m/hr, 230Volts; Zepol, S.L mod. CBB60N; Spain) introduced the air, which was evenly distributed before exiting through an opening at the top. The system was normobaric, and the pressure inside the chambers never exceeded 33 mmH₂O. Although both chambers were maintained under similar environmental conditions, only one chamber filtered the air using a series of three filters: the first two filters trapped large and medium-sized particles (metal and pleated filters; 24 × 24 × 2 cm; featuring MERV8 particle filters and a final HEPA PH97 filter that eliminated 99.97% of particles larger than 0.3 microns). The third filter was a Purafil PSA 102 device, with a capacity of 500 cfm (Purafil Inc., USA), fitted with a Purafil Select filtering medium in PK12 modules (Purafil Inc., USA).

2.3. Air analysis

The PM2.5 concentration inside the chambers was monitored daily with a digital analyzer, and outside using a beta attenuation monitor BAM 1020 (Met One Instruments, Inc., Grant Pass, OR, USA; Beta Source: 4 C (carbon-14), 60 $\mu\text{Ci} \pm 15 \mu\text{Ci}$ (2.22 MBq), equipped with a photomultiplier tube Beta Detector with an organic plastic scintillator at a flow rate of 16.7 liters per minute. Results were expressed in $\mu\text{g}/\text{m}^3$. The data was provided by "Algoritmos y Mediciones Ambientales SpA" from the "Las Encinas Monitoring Station", located 200 m from the chambers and available online at the National Air Quality Information System (<https://sinca.mma.gob.cl>). NO₂ and CO gas concentrations were consistent in both chambers, as the filtering system did not retain these pollutants.

2.4. Study design

We investigated the relationship between exposure to PM2.5 and the morphofunctional characteristics of the placenta using a crossover case design based on Veras et al. [70]. Following Chilean Law 20.380, the

Guide for the Care and Use of Laboratory Animals [46] and approved by the Scientific Ethics Committee of the Universidad de La Frontera (Record 122/20), two generations of rats were exposed to air pollution. In the development of animal procedures, we have rigorously adhered to current legislation and guidelines established for animal research. Furthermore, we have ensured compliance with the ARRIVE guidelines (Animals in Research: Reporting *In Vivo* Experiments; [50]) to ensure transparency and ethics in our animal research. Our study centered on the second generation (G2) rats to examine the effects of pre-gestational and gestational exposure to PM2.5, aiming to minimize confounding variables and inherited effects from previous exposures in earlier generations (G0). This methodology enabled us to directly isolate and assess the impact of PM2.5 exposure on the health of the placenta in G2 and the fetal size in G3, thereby offering a more precise comprehension of the health implications associated with such exposure. Four groups of first-time mothers G2 rats were continuously exposed from birth until the day of the cesarean section (21 days post-fertilization; 21-dpf). G2 rats were consistently exposed to Filtered Air (FA; n=24) and Non-Filtrated Air (NFA; n=24) from birth until the first day of pregnancy ("pre-gestational stage"). Subsequently, during the gestational stage, each group was divided into two exposure groups (n=12) ("gestational stage"). The G1 rats came from pregnancies in both chambers. Upon maturing, 24 G1 females were mated, giving rise to the G2 rats. These, upon reaching reproductive maturity (around 60 days), were evaluated using vaginal cytology and observation of preovulatory follicles. The estrous cycle stage was determined with daily vaginal cytology, and to confirm gestation, the presence of a semen plug in the

vagina was checked.

2.5. Groups

After mating, the females were divided into four study groups, defined as: FA/FA (G2 female rats that were raised and also had a full-term gestation in a chamber with an air filter), FA/NFA (G2 female rats that were raised in a chamber with an air filter and had a full-term gestation in a chamber without an air filter), NFA/FA (G2 female rats that were raised in a chamber without an air filter and had a full-term gestation in a chamber with an air filter), and NFA/NFA (G2 female rats that were raised and also had a full-term gestation in an unfiltered chamber). All G2 rats included in this study displayed at least one estrus (Fig. 1C).

2.6. Placental morphometry

Six placentas per group were randomly selected from cesarean sections (21-dpf) and fixed in 10% paraformaldehyde for 24 h. The total placental volume (P_{TV}) was determined using the Cavalieri Principle with the ImageJ software [58]. Cross-sectional images of the placenta were acquired with a HITACHI SU 3500 Scanning Electron Microscope at a 133 eV resolution on the MnK α line. In rats, the placenta consists of a fetal part, which includes the labyrinthine (ZL) and basal (ZB) zones, and a maternal part, represented by the decidua (ZD) [59]. The ZL, where maternal-fetal oxygen exchange occurs, is highly vascularized and contains trophoblastic septa and maternal sinusoids [17,8].

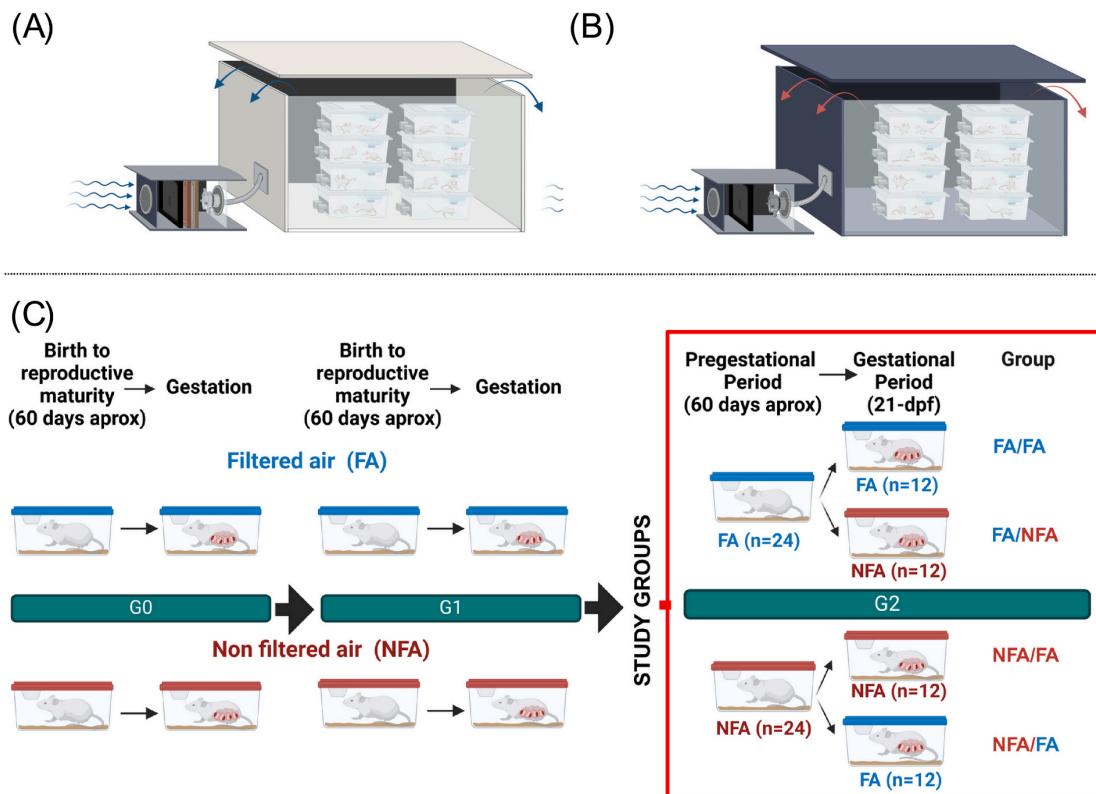


Fig. 1. Experimental design. (A) Filtered air chamber. (B) Non-filtered air chamber. (C) Exposure groups. We investigated the relationship between exposure to PM2.5 and the morphofunctional characteristics of the placenta in rats using a crossover design. Two generations of rats were used, focusing on the second generation (G2) to assess the effects of both pre-gestational (birth to reproductive maturity) and gestational exposure (21 days post-fertilization). These G2 rats were divided into four groups, based on their exposure to filtered air (FA) or non-filtered air (NFA) during the pre-gestational and gestational phases: FA/FA, FA/NFA, NFA/FA and NFA/NFA. The G1 rats gave rise to the G2 rats, and once the latter reached reproductive maturity, they were monitored and grouped based on their environmental exposure and reproductive status.

Volumetric and planimetric analysis of the placental compartments was conducted with calibrated digital images of cross-sections using ImageJ. Results were expressed as total volume (V_T ; cm^3), total surface area (S_T ; cm^2), and area proportion (%). Stereological analysis in the ZL was conducted using the STEPanizer Stereological Tool [68], applying a point-counting planimetric system (M36 Test System) on digital images (Mayhew et al., 2009). The variables quantified included: I) fetal capillary, II) maternal blood space, and III) trophoblast. The volume density (VV%) of fetal capillaries, maternal blood space, and trophoblast was estimated through point counting.

2.7. Physical analysis of placenta

The thickness of the interhemal membrane in the ZL was calculated by overlaying digital images on a grid planimetric system with an area per point of 80,000 pixels² using ImageJ software. This grid served to determine starting points for measuring the distance between the maternal-fetal space and the nearest capillary. For each fetal capillary intercepted by the grid, the distance (cm) between the maternal-fetal space and the closest capillary was measured. The harmonic mean thickness (Th) was calculated using the reciprocal of the average of the inverted distances and corrected with the factor $8/(3\pi)$ ([9]a). The theoretical diffusion capacity (Dm) of the interhemal membrane ($\text{cm}^3 \text{min}^{-1} \text{kPa}^{-1}$) was determined by multiplying Krogh's diffusion coefficient (K) for oxygen ($17.3 \times 10^{-8} \text{ cm}^2 \text{ min}^{-1} \text{kPa}^{-1}$) [35] and the average between the surface of the maternal-fetal space and the fetal capillary, divided by the harmonic mean thickness (Th). The specific diffusion capacity ($\text{cm}^3 \text{min}^{-1} \text{kPa}^{-1} \text{g}^{-1}$) was estimated by the quotient of the theoretical diffusion capacity of the interhemal membrane over the fetal weight.

2.8. Immunohistochemistry

Six placentas were randomly selected from each exposure group, fixed in 10% formaldehyde, and embedded in paraffin. The dehydrated samples were cut into 6 μm sections. These were deparaffinized with histoclear and then passed through a series of alcohols in decreasing concentrations. They were incubated with specific antibodies: anti-VEGF-A (1:200, ab231260, Abcam, Cambridge, UK), anti-PIGF (1:200, ab230526, Abcam, Cambridge, UK), Flt-1 (anti-VEGF-R1; 1:50, ab2350, Abcam, Cambridge, UK), Kdr (anti-VEGF-R2; 1:50, ab2349, Abcam, Cambridge, UK), and anti-HIF-1 α (1:100, sc13515, Santa Cruz, Dallas, TX, USA) overnight at 4°C. Detection was performed using the IHC Select® HRP/DAB kit (DAB150, Merck), following the manufacturer's instructions. They were observed and photographed under a Leica® DM750 microscope with a Leica® MC170HD camera. Quantification was performed using the ImageJ software, presenting the results as a fraction of the area (%).

2.9. RNA extraction and cDNA synthesis

Three placentas per exposure group were randomly selected, preserved in RNA Later® (Invitrogen, Carlsbad, CA, USA) post Cesarean, and stored at -80°C. RNA was purified combining TRIzol® (Invitrogen, Carlsbad, CA, USA) and the E.Z.N.A Total RNA kit (Omega Biotech Inc., Atlanta, USA). The tissue was solubilized and homogenized in 750 μl of TRIzol®, and after an incubation, 250 μl of chloroform was added, followed by centrifugation at 12,000 x g for 10 min at 4°C. The aqueous phase was recovered and mixed with 700 μl of absolute ethanol. Using the E.Z.N.A Total RNA kit, samples were purified following the manufacturer's instructions. RNA quantification was performed using a Nandrop-1000, and the samples were stored at -80°C until further use.

2.10. qRT-PCR analysis

mRNA of genes related to angiogenic growth factors and HIF-1 α was quantified. The qPCR assays, conducted with a volume of 20 μl , included: 1X KAPA SYBR® FAST Universal qPCR Master Mix (Merck, Darmstadt, Germany), primers (Table 1) at 200 nM, and 1 μl of template cDNA. The amplification and detection of genes were carried out on a CFX96 real-time PCR system (Bio-Rad Laboratories Inc., Hercules, CA, USA) under the following conditions: I) 95°C for 3 min, II) 40 cycles of 95°C for 15 seconds, 58°C for 15 seconds, and 60°C for 20 seconds; III) melt curve from 65°C to 90°C, increasing by 0.5°C every 5 seconds. Data were processed with the CFX Manager 2.2.1 (Bio-Rad Laboratories Inc., Hercules, CA, USA). Expressions of VEGF-A, PIGF, Flt-1, Kdr, and HIF-1 α were analyzed. The ACT-B gene, corresponding to beta-actin, was used as the housekeeping gene. Expression analyses were obtained using the 2- $\Delta\Delta\text{CT}$ method [40].

2.11. Statistical analysis

All data were organized in Excel spreadsheets and expressed as mean \pm standard deviation. After applying the D'Agostino-Pearson Normality Test to verify the distribution, a one-way ANOVA or Kruskall-Wallis test was used, depending on whether the distribution was parametric or not. Differences between groups were identified with post hoc tests (Tukey or Dunnett). A p-value of <0.05 was considered significant, with a 95% confidence interval. Analysis was conducted using GraphPad Prism 9.0 for Mac OS (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. Environmental exposure

During the experimental stage, the average daily levels of ambient air for PM2.5, PM10, and CO were $48.8 \mu\text{g/m}^3 \pm 36.1$ (CV=74%), $56.9 \text{ mg/m}^3 \pm 38.3$ (CV=67.3%), and $0.78 \text{ ppm} \pm 0.49$ (CV=61.5%), respectively (Table 2). In the NFA chamber, the average concentration of PM2.5 was $44.6 \mu\text{g/m}^3$, similar to the environmental measurements. In contrast, the FA chamber displayed a significantly lower average concentration of $3.0 \mu\text{g/m}^3 (\pm 1.3; \text{CV}=34.3\%; p<0.001)$, reflecting a 94% reduction compared to the NFA chamber. The values during the study period were higher because this period corresponded to winter in the southern hemisphere, which is associated with an increase in the consumption of firewood for residential heating. To estimate maternal exposure, the total volume inhaled during gestation (1299 liters, based on 22 days and an inhalation volume of 0.041 l/min) was multiplied by the average 24-hour PM2.5 concentration. Thus, NFA/NFA females inhaled an estimated total of 57934 mg/m^3 and 2633.40 mg/m^3 per day, whereas FA/FA females inhaled 3896.97 mg/m^3 in total and 177.14 mg/m^3 daily.

3.2. Maternal and fetal morphometry

The data presented in Fig. 3A demonstrate the effect of PM2.5 on maternal and fetal morphometry at 21-dpf. The groups showed variations in maternal and fetal weight, as well as in fetal crown-rump length (CRL), which could be attributed to exposure to PM2.5 during gestation. There is a clear trend toward lower maternal weight ($p=0.0615$), indicating that exposure to PM2.5 had some effect (although not statistically significant) on mothers' weight at 21 dpf. There is a clear trend towards lower maternal weight ($p=0.0615$), indicating that exposure to PM2.5 did not have a measurable impact on maternal weight at 21-dpf. The average maternal weight ranged between $268.88 \pm 14.98 \text{ g}$ in the FA/NFA group and $309.95 \pm 28.18 \text{ g}$ in the FA/AF group. In contrast, fetal weight showed significant differences between groups ($p=0.0022$), with the FA/FA group presenting an average fetal weight 12% higher ($5.09 \pm 0.60 \text{ g}$) compared to the NFA/NFA group ($4.53 \pm 0.59 \text{ g}$) (Fig. 3B). The

Table 1

Primers used for gene expression evaluation.

GEN	PRIMER FORWARD (5' - 3')	PRIMER REVERSE (5' - 3')	REFERENCE
VEGF-A	5'- CAGCACATAGGAGAGATGAGCTTC-3'	5'- GTCTCGGGATCTGGACAAA-3'	[11]
FLT1	5'-CAGAGCCAGGAACATATAACAGG-3'	5'- CGTGGTCACTGAGGTTTGAAAG-3'	[11]
KDR	5'-CTAGTCAAGCAGCTCGTCATCCA-3'	5'- GCCTGAATCTTACAAGGGCTC-3'	[11]
PIGF	5'- ATGTGTCCTCTGAGTCGCTGTAG-3'	5'-CTGCCCTGCTCTCCAGAACATAG-3'	[11]
ACT-B	5'-ACCCGCGAGTACAACCTCT-3'	5'- GCAGCGATATCGTCATCCAT-3'	[11]
HIF-1 α	5'-CAACTGCCACCAGTGTGAA-3'	5'-TGGTAGAAGGTGAGATGC-3'	[33]

Table 2

Average daily levels of PM2.5, PM10, and CO in ambient air.

Levels of PM2.5, PM10 and CO			
Pollutant	Study period	Peak 2021	Annual 2021
PM _{2.5} [ug/m ³]	48.8 ± 36.1 (CV: 74%)	157 12 events	26.2 ± 30.6 (CV: 117%)
		>100 ug/m ³	
PM ₁₀ [ug/m ³]	56.9 ± 38.3 (CV: 67.3%)	182 20 events	36.6 ± 30.8 (CV: 84.2%)
		>100 ug/m ³	
CO [ppm]	0.78 ± 0.49 (CV: 61.5%)	2.45 ppm 3 events > 2 ppm	0.44 ± 0.43 (CV: 97.7%)

PM 2.5: Fine particulate matter >2.5 μ m; PM 10: Fine particulate matter >10 μ m; CO: Carbon monoxide.

FA/NFA group had the lowest average fetal weight (4.11 ± 0.44 g), suggesting a possible detrimental effect of PM2.5 exposure during gestation. Furthermore, fetal crown-rump length (CRL) also showed significant differences ($p=0.0005$), with the FA/FA group having the highest average CRL (39.25 ± 2.11 mm), significantly longer than the NFA/NFA group (35.23 ± 4.75 mm). This reinforces the notion that exposure to PM2.5 during gestation impacts optimal fetal development, as indicated by both weight and CRL metrics. These findings suggest that the quality of ambient air during critical periods of fetal development has a measurable impact on morphometric outcomes. Exposure to PM2.5 during gestation appears to affect fetal growth parameters, as demonstrated by the increased weight and CRL in the FA/FA group (Fig. 2A, Fig. 3C).

3.3. Placental morphometry

The FA/FA group, not exposed to PM2.5, showed a higher Total Placental Volume (cm³) compared to groups exposed to PM2.5 either in the pre-gestational and/or gestational stages (FA/NFA, NFA/FA, NFA/NFA) (Fig. 2B-2D, Fig. 3D). Regarding the volumes (cm³) of placental compartments, there were notable differences in the ZD volumes between FA/NFA and NFA/FA ($p=0.0017$) (Fig. 3E). Moreover, groups exposed to PM2.5 had lower average volumes of both ZB and ZL compared to FA/FA, with the differences being significant for ZB ($p<0.0001$) and ZL ($p=0.0049$) (Fig. 3F-G). As for the sub-compartments of the ZL, exposure to PM2.5 was associated with lower total volumes (V_T) of fetal capillaries ($p<0.0001$) (Fig. 3H), V_T of the materno-fetal space ($p<0.0001$) (Fig. 3I), V_T of trophoblastic tissue ($p<0.0001$) (Fig. 3J), and the total surface area (S_T) of the materno-fetal space ($p<0.0001$) (Fig. 3L), compared to the unexposed FA/FA group. This latter group also showed a higher average S_T of fetal capillaries, which was significantly different ($p=0.0039$) (Fig. 3K) from groups exposed only in the pre-gestational stage (NFA/FA and NFA/NFA).

3.4. Physical analysis of placenta

Both groups, FA/FA and NFA/NFA, exhibited an intricate network of trophoblastic septa. However, while the FA/FA group displayed a cytотrophoblast with prominent nuclei and nucleoli, suggesting high metabolic activity, and a dense array of fenestrated fetal capillaries, the

NFA/NFA group showed a lower density of both maternal sinusoids and fetal capillaries. Despite these differences, in both groups, expansive maternal-fetal spaces were observed, allowing unrestricted maternal blood flow, promoting close contact with the trophoblastic epithelium and fetal capillaries. It is noteworthy that in both cases, the maternal sinusoids are filled with blood and lack an endothelial barrier, facilitating maternal-fetal exchange. Concerning the diffusion capacity of the interhemal membrane, the FA/FA group, not exposed to MP2.5, displayed significant differences compared to the exposed groups, either before or during gestation (FA/NFA, NFA/FA, NFA/NFA). Specifically, the unexposed FA/FA showed significant differences in harmonic mean thickness ($p<0.0001$) (Fig. 3M), theoretical diffusion capacity ($p<0.0001$) (Fig. 3N), and specific diffusion capacity ($p<0.0001$) (Fig. 3O). Notably, FA/FA exhibited approximately double the harmonic mean thickness and a reduced diffusion capacity, both theoretical and specific, compared to NFA/NFA (Fig. 2E-F).

3.5. Angiogenic factors and HIF-1 α in placenta

The immunohistochemistry images of angiogenic factors and HIF1 α in the placenta can be found in [supplementary information 2](#). Based on the quantification of angiogenic factors and HIF-1 α using qPCR, no differences were observed in the expression of VEGF-A among the groups ($p=0.4167$; Fig. 4B). However, an increase in positive reaction was observed, primarily in the ZB, in groups exposed to PM2.5 during the gestational stage. Exclusive exposure to PM2.5 in the pre-gestational phase boosted the expression of PIGF, Flt-1, Kdr, and HIF-1 α in the placenta. There were differences in the expression of PIGF between FA/FA and FA/NFA ($p<0.0001$; Fig. 4A), with an increase in the positive reaction noted across the three placental compartments. The Flt-1 receptor showed significant differences ($p<0.0001$) between the FA/NFA group, and the groups exposed to MP2.5, in both pre-gestational and gestational stages (NFA/FA, NFA/NFA; Fig. 4C). Specifically, the NFA/FA group displayed a notable rise in the expression of Flt-1 across all three placental compartments. Similarly, the Kdr receptor exhibited significant differences among all exposure groups ($p<0.0001$; Fig. 4D). An increase in positive reaction was noted in groups not exposed to PM2.5 during the gestational phase. Concerning HIF-1 α , significant differences were observed ($p<0.0001$; Fig. 4E) between the FA/FA group and those exposed to PM2.5 during the pre-gestational and/or gestational stages. The NFA/FA group revealed a significant rise in the expression of HIF-1 α in the ZL and ZB.

Based on the determination of the area with positive IHC staining, an increase in the expression of PIGF ($p<0.0001$; Fig. 5A), VEGF-A ($p<0.0001$; Fig. 5B), Flt-1 ($p<0.0001$; Fig. 5C), Kdr ($p<0.0001$; Fig. 5D) and HIF-1 α ($p<0.0001$; Fig. 5E) was detected in groups exposed to PM2.5. Endothelial cells showed an increase in positive reaction to VEGF-A (Fig. 5G) in groups exposed to PM2.5 during the pregestational and/or gestational stages, as well as the expression of Flt-1 (Fig. 5H) and Kdr (Fig. 5I). The reaction to PIGF and HIF-1 α was intense across all exposure groups. However, there was an observed increase in positive endothelial reaction to PIGF (Fig. 5F) and HIF-1 α (Fig. 5J) when the exposure was exclusively during the pregestational stage. In the trophoblastic tissue, exposure to PM2.5 during gestation showed an increase in the number of syncytiotrophoblasts and cytotrophoblasts

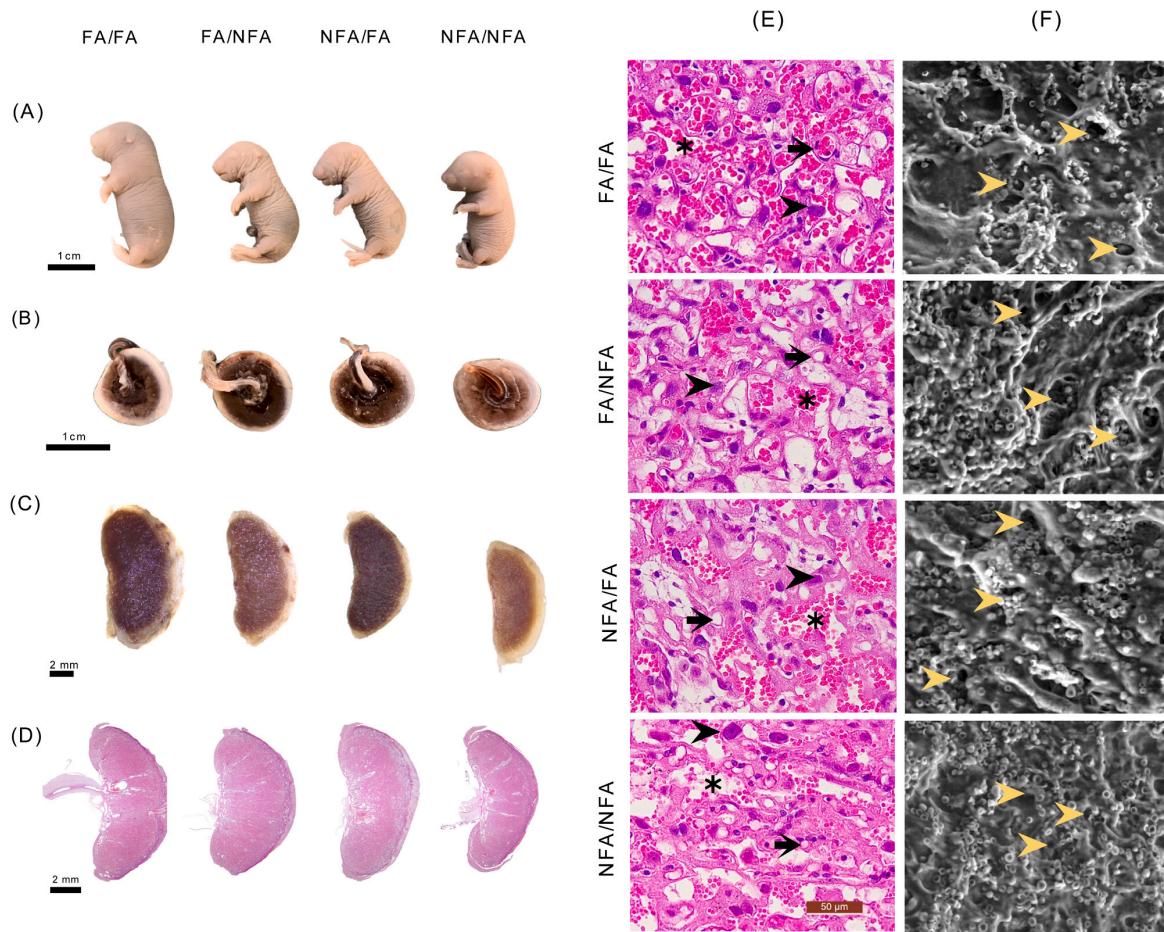


Fig. 2. Effects of exposure to PM2.5 during pregestational and/or gestational stages on fetal and placental morphology. (A) G3 fetuses at 21 days post-fertilization, showing a reduction in placental volume and its compartments in groups exposed to PM2.5. (B) View of the fetal surface of the placenta and the umbilical vessels. (C) Cross-section of the placenta. (D) Detail of a placental cross-section stained with hematoxylin and eosin, indicating an increase in the thickness of the trophoblastic tissue, leading to a thickening of the interhemal membrane and a subsequent decrease in the oxygen diffusion capacity in the labyrinth zone. (E) Placenta, labyrinth zone (blue arrow: fetal capillary; red arrow: maternofetal space; black arrow: trophoblast) (40x magnification) stained with hematoxylin and eosin. (F) Placenta, labyrinth zone observed by scanning electron microscopy (yellow arrow: fetal capillary). FA: filtered air, NFA: non-filtered air.

that exhibited a positive reaction to PIGF (Fig. 5F), VEGF-A (Fig. 5G), Flt-1 (Fig. 5H), and HIF-1 α (Fig. 5J) in those groups exposed to PM2.5, whether in the pregestational or gestational phase. Nevertheless, the response to Kdr in the trophoblastic tissue was faint, regardless of the exposure group (Fig. 5I).

4. Discussion

This study investigates the effects of exposure to wood smoke on placentas and fetuses, highlighting a reduction in fetal size associated with maternal exposure to wood smoke both before and during gestation. The analysis identifies a trend towards decreased fetal dimensions when the mother has been exposed to wood smoke, suggesting that such exposure has a detrimental impact on fetal development. This reduction is attributed to changes in angiogenesis induced by prolonged hypoxia in the placenta, affecting the oxygen supply to the fetus. This research is pioneering in analyzing the effects of PM2.5 derived from wood combustion on placental and vascular health in pregnant rats in a highly polluted region: Temuco, Chile, where PM2.5 levels exceed health guidelines. The findings reported in this study show that PM2.5 exposure induces the expression of angiogenic factors like HIF-1 α and VEGF-

A in response to hypoxia. Pregestational exposure to PM2.5 increases the expression of PIGF, Flt-1, and Kdr, accompanied by changes in vascular structure. The placenta's adaptation to hypoxia has clear effects on angiogenesis and the oxygen diffusion capacity to the fetus. This adaptation leads to changes in vascular morphology and an increase in the trophoblastic tissue of the placenta, which compromises the efficiency of oxygen exchange between mother and fetus. These factors lead to reductions in weight and cranio-caudal length of fetuses exposed to PM2.5 during the pregestational and/or gestational stages.

Based on our knowledge, this is the first time that the impact of pregestational and/or gestational exposure to PM2.5, from wood burning smoke, has been evaluated on the placenta and blood vessels that form part of the placental. The concentration and duration of PM2.5 exposure during the study period were clearly defined, highlighted as a key aspect since they are crucial variables for understanding the dose-response relationship between exposure and the observed effects. During the exposure period, the average PM2.5 was $48.8 \mu\text{g}/\text{m}^3$ (± 36.1 ; CV=74%), tripling the daily limit recommended by the WHO ($15 \mu\text{g}/\text{m}^3$, 2021).

At the end of the gestational period (21 days post-fertilization), no differences in the weight of the pregnant mothers were observed,

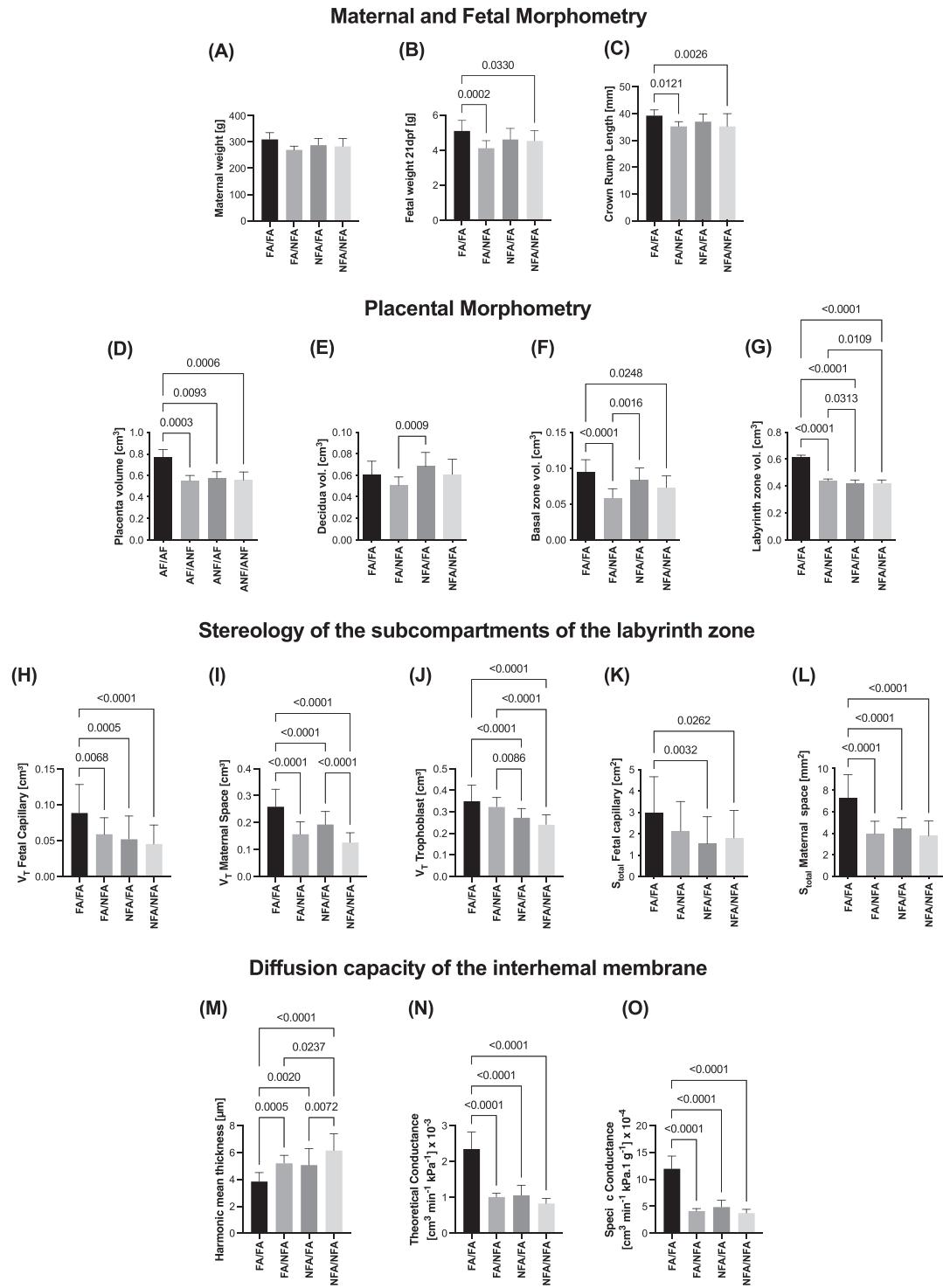


Fig. 3. Maternal, Fetal, and Placental Morphometry and Interhemal Membrane Diffusion Capacity. A-C illustrate maternal and fetal morphometry at 21 days post-fertilization (dpf): (A) Maternal weight at the end of the study period, (B) Fetal weight, and (C) Crown-rump length (CRL) of the fetuses. D-G display placental morphometry parameters: (D) Placental volume, (E) Decidua volume, (F) Basal zone volume, and (G) Labyrinth zone volume. H-L describe the stereology of the subcompartments of the labyrinth zone: (H) Volume density of fetal capillaries, (I) Volume density of maternal space, (J) Volume density of trophoblast, (K) Surface density of fetal capillaries, and (L) Surface density of maternal space in the labyrinth zone. M-O present the diffusion capacity of the interhemal membrane: (M) Harmonic mean thickness, (N) Theoretical Conductance, and (O) Specific Conductance. Each bar represents the mean (\pm SD). Statistical analyses were performed using ANOVA with Tukey's posthoc to identify significant differences between groups.

GENE EXPRESSION

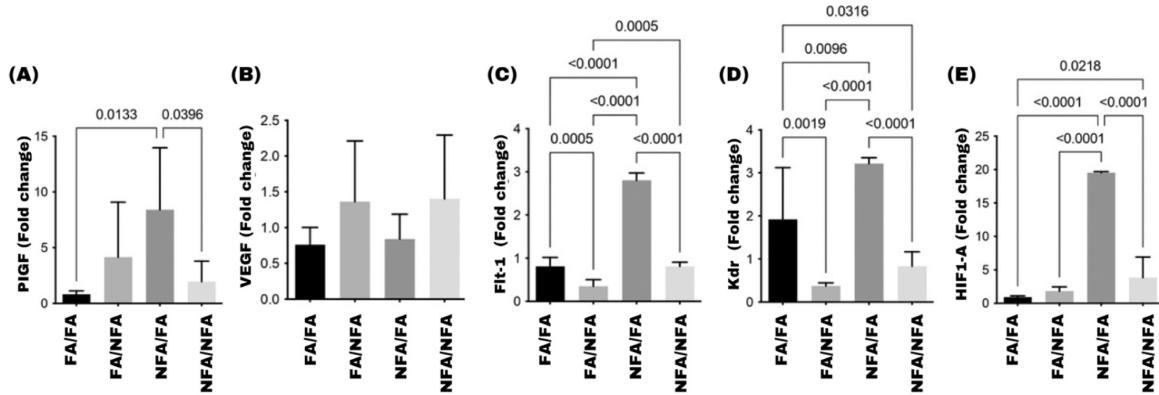


Fig. 4. Quantification of gene expression by qPCR for (A) PIGF, (B) VEGF-A, (C) Flt-1, (D) Kdr, and (E) HIF1- α . Lines above the bars indicate statistically significant differences between groups ($p < 0.05$). FA: filtered air, NFA: non-filtered air. Each bar represents the mean (\pm SD). Statistical analyses were performed using ANOVA with Tukey's posthoc to identify significant differences between groups.

suggesting that exposure levels of $48.8 \mu\text{g}/\text{m}^3$ of PM2.5 did not influence maternal growth, at least at the end of the gestational stage. However, a reduction in fetal weight and crown-rump length (CRL) was detected in groups exposed to PM2.5 during the pregestational or gestational stages. This finding indicates that PM2.5 exposure affected fetal size not only during gestation but also that prior exposure had consequences, suggesting an influence of prior maternal exposure which could include a systemic effect localized in the uterine environment before the gestational stage, leading to long-term alterations. In humans, various studies [24,36,76] have shown that birth weight at term (≥ 37 weeks of gestation) was negatively associated with maternal exposure to PM2.5 during gestation. Even exposure levels to PM2.5 lower than $10 \mu\text{g}/\text{m}^3$ had adverse effects on term birth weight. Although with high heterogeneity, two previous meta-analyses indicated that maternal (*i.e.*, prenatal) exposure to ambient PM2.5 could increase the risks of low birth weight (Dadvand et al., 2013a; Li et al., 2020). The reduction in average fetal weight and CRL observed during exposure to wood smoke (PM2.5: $48.8 \mu\text{g}/\text{m}^3$) was also reported in studies where the source of PM2.5 derived from petroleum combustion in motor vehicles and industries [14,37,55,69,70]. This underscores the importance of considering different pollution sources when assessing the risks associated with air quality and its impact during the gestational stage.

Reduced fetal weight was associated with a decreased placental volume. Furukawa et al. [18] claimed that the placenta reflects changes in the ZL induced by exposure to various factors throughout gestation, particularly during the last two-thirds. These changes in the ZL might be linked to a delay in placental growth, since this compartment increases its volume towards the end of gestation due to vascular remodeling, allowing increased blood flow between the mother and the fetus [9]. This vascular remodeling, evident in volume and surface area, is essential for optimal oxygen transport and directly influences fetal size. Mayhew [44] contends that this vascular transport is proportional to fetal growth, as vessels typically adapt by expanding their surface area, ensuring that the placenta's diffusion capacity matches fetal growth [44]. On the other hand, prior exposure to PM2.5 appears to alter this adaptation, increasing the volume of the ZD and reducing maternal capillaries. This suggests that the alterations detected in maternal vasculature might arise due to systemic disorders or a previous compromise in the endometrial environment.

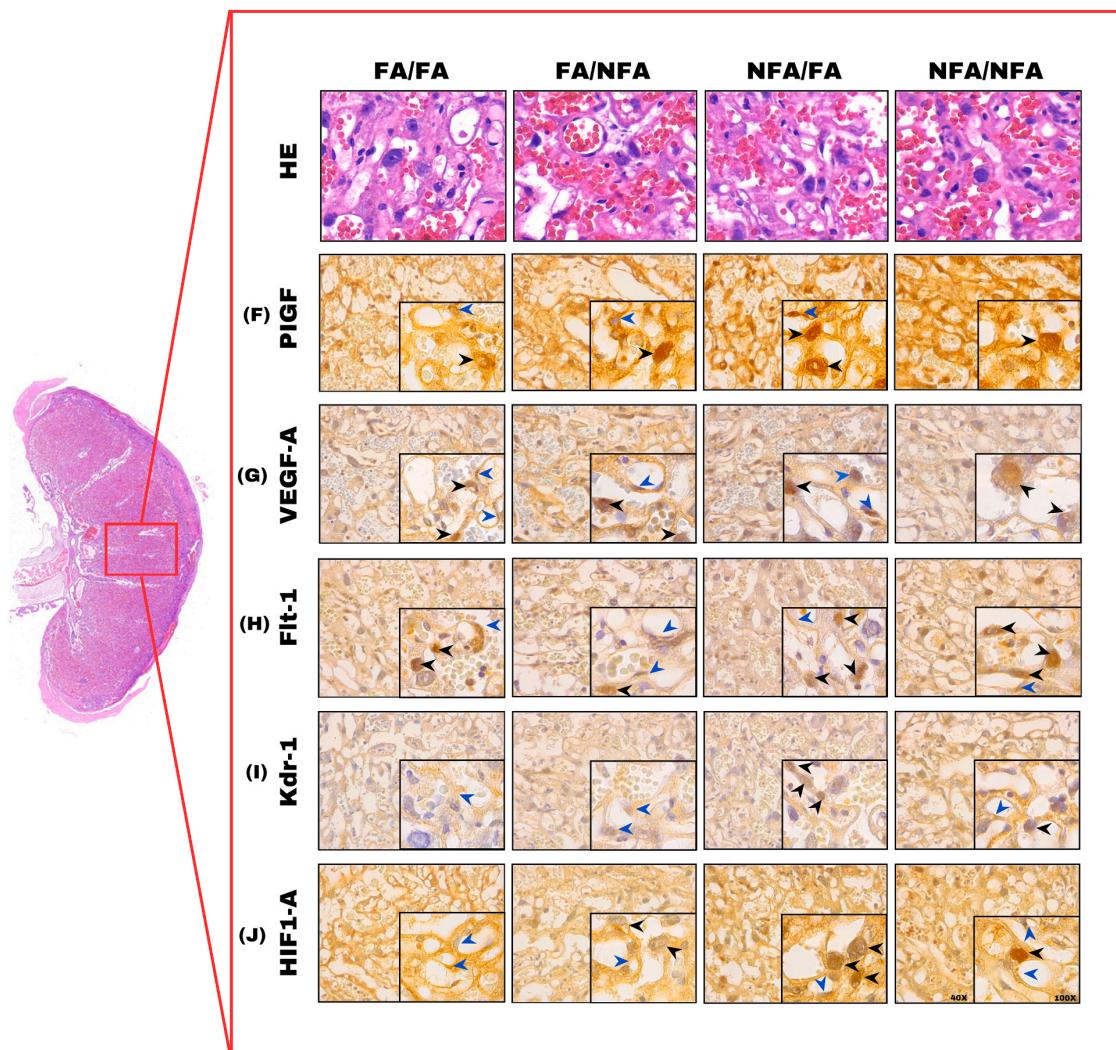
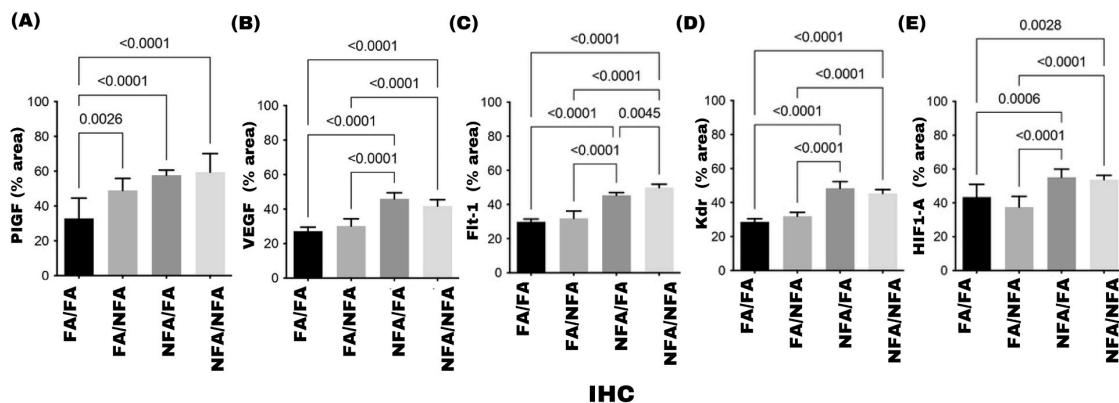
Exposure to PM2.5 during pregestational and/or gestational stages was linked to a decrease in volume and surface area of fetal capillaries and an increase in trophoblastic tissue, leading to a thickening of the interhemal membrane. These findings are consistent with a previous

study in rats, where PM2.5 exposure resulted in developmental anomalies, including reduced surface area, imbalances in placental angiogenic factors, and alterations in trophoblast differentiation [66]. It's worth noting that an increase in trophoblastic tissue can compromise oxygen diffusion, lengthening the diffusion distance between the mother and fetus, which raises the risk of a fetal hypoxic stress scenario ([45]; Auad et al., 2020). Structurally speaking, a reduction in the ability to diffuse oxygen through the interhemal membrane induces a state of hypoxia. This diffusion capacity is related to trophoblastic tissue differentiation [65], which is strongly regulated by hypoxic conditions during the early stages of gestation. Oxygen tension is crucial for early placental development and trophoblast differentiation [62,63]. Under hypoxic conditions, trophoblastic cells do not differentiate properly. However, an increase in O_2 concentrations reverses this effect, as indicated by Sallais et al. [56]. Sustained hypoxia can lead to deterioration in the quantity and quality of trophoblastic cells, jeopardizing fetal health and hindering vascular remodeling in the placenta ([65]; Valenzuela et al., 2022). Under normal conditions, the thickness of the interhemal membrane decreases as maternal and fetal vascular surfaces expand, increasing the exchange area and reducing the diffusion distance, thus facilitating the exchange of nutrients and gases [9].

In our study, we observed an increase in the thickness of the interhemal membrane, correlated with a reduction in the surface area of blood vessels and compromised diffusion. This change negatively impacts oxygen exchange, reducing the amount available to the fetus. The volumetric and planimetric data of the placenta obtained in this study are consistent with findings from previous research on murine placentas [48,54,64,72,9]. While there was agreement in areas such as volume and proportions of the maternal-fetal space, discrepancies were noted, possibly due to tension variations. The most notable differences were observed in the arithmetic mean thickness, the caliber of the vessels, and diffusive conductances. These variations might be attributed to differences in the animal model or estimation methods. Nonetheless, our results provide consistent comparisons between different experimental groups. Therefore, it's important to note that results derived from arithmetic thickness calculations should be interpreted with caution.

Oxygen adaptations are essential for proper placental development [56]. In our study, pregestational exposure to PM2.5 increased the expression of the HIF-1 α factor, demonstrating an adaptive response during late gestation, apparently prolonging the condition of physiological hypoxia observed during early gestation [6]. In hypoxic environments, HIF-1 α is intensified, promoting the release of VEGF-A and stimulating angiogenesis [34]. This statement aligns with our findings.

PROTEIN LEVELS



(caption on next page)

Fig. 5. Quantitative assessment of immunostaining by area fraction analysis for (A) PIGF, (B) VEGF-A, (C) Flt-1, (D) Kdr, and (E) HIF1- α . Lines above the bars indicate statistically significant differences between groups ($p < 0.05$). Increased immunohistochemical staining in trophoblastic tissue for PIGF, VEGF, Flt1, Kdr, and HIF1- α in the region of the placental labyrinth zone associated with exposure to PM2.5 during the pregestational and/or gestational stages. (F) Intense positive signaling for PIGF in the endothelium of specimens exposed to PM2.5 during pregestational and/or gestational stages. An increase in positive staining for (G) VEGF-A, (H) Flt-1, and (I) Kdr was observed in endothelial cells of groups exposed to PM2.5 in pregestational and/or gestational stages. (J) Strong positive signaling for HIF-1 α in specimens exposed to PM2.5 in the pregestational stage. In the trophoblastic tissue, a higher number of syncytiotrophoblasts and cytotrophoblasts with positive reactions for (F) PIGF, (G) VEGF-A, (H) Flt1, and (J) HIF-1 α were detected in groups subjected to exposure to PM2.5 in the pregestational and/or gestational stages. (I) Signaling for Kdr did not show intensity in the trophoblastic tissue and was independent of the exposure group (blue arrow: endothelial cells; gray arrow: trophoblastic tissue). FA: filtered air, NFA: non-filtered air. Each bar represents the mean (\pm SD). Statistical analyses were performed using ANOVA with Tukey's posthoc to identify significant differences between groups.

However, sustained hypoxia can lead to delayed placental responses affecting hormonal regulation, nutrient absorption, and the release of proangiogenic factors [10]. While the expression of VEGF-A, associated with the hypoxic environment, did not show significant differences in expression among the evaluated groups, it did exhibit a biologically relevant increase in expression in the placenta of groups exposed to PM2.5 during the gestational stage. This increase in VEGF-A seems to be relevant for vascular remodeling and oxygen supply to the fetus under hypoxic conditions. Furthermore, exposure to PM2.5 during the pregestational stage increased the expression of PIGF, Flt-1, and Kdr. Zhang et al. [75] administered PM2.5 via nasal instillation to 8-week-old rats and reported that prior exposure to PM2.5 altered angiogenesis and uterine structure in rats, leading to abnormal vascular remodeling post-gestation. We believe that the increase in angiogenic factors is also an adaptive physiological response during late gestation to exposure to PM2.5. In addition, the increase of Flt-1 (vascular endothelial growth factor receptor 1, VEGF) plays a crucial role in modulating the activity of VEGF-A, a key factor in the angiogenesis process. However, Flt-1 not only captures VEGF-A, limiting its availability to interact with other receptors such as VEGFR-2 (KDR/Flk-1), but it can also form complexes with these other receptors, thus modulating VEGF-A signaling and its impact on angiogenesis [43]. Despite the high expression of PIGF, Flt-1, its antagonist, is also overexpressed. It is known that PIGF increases in hypoxic environments and has affinity for Flt-1 [30]. Moreover, the elevated expression of Kdr could potentiate the binding of VEGF-A, thus counteracting the action of Flt-1 [38].

In humans, the augmented expression of HIF-1 α and alterations in angiogenic factors are pivotal in sustaining placental functionality and fetal development in polluted settings. Nonetheless, persistent hypoxia and disrupted angiogenesis may have enduring consequences on fetal growth and health [62,63,7], highlighting the necessity for additional investigations in this domain. Similar to observations in rats, the human placenta adjusts its expression of angiogenic factors and their receptors in reaction to hypoxia. VEGF and PIGF both exhibit an upsurge in expression under hypoxic conditions. These factors engage with their specific receptors, Flt-1 and KDR, to facilitate adaptive cellular responses. Hypoxia enhances the expression of VEGF, Flt-1, and KDR in trophoblastic cells and placental tissues, demonstrating an adaptation aimed at fostering angiogenesis and vascular development within the hypoxic placental milieu [1,67]. Furthermore, PIGF has been identified to bind with high affinity to Flt-1 but not to Flk-1/KDR, indicating that PIGF and VEGF may serve complementary yet distinct roles in placental angiogenesis, with PIGF augmenting the bioactivity of VEGF through its interaction with Flt-1 [49]. This differential regulation of VEGF, PIGF, and their receptors in response to hypoxia underscores the intricacy of the molecular adaptations of the human placenta to ensure adequate fetal development under adverse conditions.

Findings in rats revealed impacts on fetal growth parameters (reduction in fetal weight and crown-rump length), changes in the placenta's oxygen diffusion capacity, and in the expression of hypoxia-inducible factor 1-alpha (HIF-1 α) and several angiogenic factors (Flt-1, Kdr, and PIGF). These findings raise concerns about similar risks in humans. However, various factors must be considered, including differences in placental structure between humans and rats, as well as the relevance of these findings in the context of human exposure to air

pollution. Both in humans and rats, the placenta is of the hemochorial type, characterized by the direct contact of trophoblastic cells with maternal blood, dispensing with an intermediary endothelium [60]. Nonetheless, fundamental differences between the two species exist. The human placenta is of the hemomonochorial type, which involves a single layer of trophoblastic cells mediating between maternal and fetal circulations. Far from being independent, these cells form a syncytium, operating together as a singular unit known as the syncytiotrophoblast [17,20]. On the other hand, the rat placenta is termed hemochorial, as it possesses three layers of trophoblastic cells between the maternal and fetal circulation. The outer layer of trophoblasts (in contact with maternal blood) is cellular, yet the middle and inner layers are syncytial [17, 2019]. Despite these differences, both placentas facilitate the exchange of nutrients and gases between the mother and fetus [19]. The effects observed in the rat placenta, particularly in terms of reduced oxygen diffusion capacity and altered expression of angiogenic factors, suggest potential risks for human placental function under similar PM2.5 exposure conditions. However, the impacts and specific mechanisms may vary. The reduction in fetal growth parameters and alterations in placental function observed in rats exposed to wood smoke could translate into similar developmental and health issues reported in humans exposed to petroleum combustion smoke [12,70]. This is particularly concerning in areas where wood burning is a common source of heating and cooking, potentially exposing pregnant women and their fetuses to elevated levels of air pollution.

The findings reported here underscore the significance of air quality management and the reduction of PM2.5 emissions, especially in areas where wood smoke substantially contributes to air pollution. Public health initiatives aimed at reducing PM2.5 exposure among pregnant women could help mitigate risks to fetal development and placental health. Further research is needed to directly assess the impacts of PM2.5 exposure on human pregnancy outcomes and explore the underlying mechanisms of action. Nevertheless, the current study adds to a growing body of evidence indicating the potential risks of air pollution on reproductive success and highlights the importance of environmental health interventions to protect pregnant women and their fetuses from the harmful effects of PM2.5 exposure.

Among the limitations of this study, it is relevant to highlight that other significant variables impacting fetal development and placental health, such as maternal nutrition, exposure to other pollutants, and the stress level in rat mothers, were not considered. Although changes in the expression of angiogenic factors were identified, the research does not delve deeply into the physiological mechanisms that could provide a greater understanding of these findings. Furthermore, the evaluation of possible variability in responses among different subjects or populations has been overlooked, which could question the universal applicability of the results. The study also focuses on the effects observed during gestation without exploring the potential long-term consequences for the offspring and mothers postpartum. Regarding the use of rats as a study model, although direct extrapolation to humans presents challenges due to physiological differences between species, this approach offers important insights into the possible impacts of PM2.5 exposure on placental function and fetal development.

In conclusion, this study reveals the impact of PM2.5 exposure, originating from wood combustion for indoor heating during pre-

gestational and gestational stages, causes alterations in the expression of angiogenic factors and decreases diffusion capacity in the interhemal membrane in the ZL, as a result of a prolonged state of placental hypoxia, affecting fetal size. In light of our research, it becomes clear that the repercussions of wood smoke pollution on public health, particularly PM_{2.5}, are profound. Reducing or avoiding exposure to this form of pollution, especially during the pre-gestational and gestational stages, emerges as an essential preventive strategy. This underscores the urgent need for proactive interventions and awareness campaigns targeting both the general population and those planning pregnancies. The silver lining here is that these adverse health outcomes, stemming from wood smoke pollution, are theoretically preventable. This demand coordinated efforts by policymakers, healthcare providers, and communities to ensure safer environments for current and future generations.

Ethics approval

Scientific Ethics Committee of the University of La Frontera (Acta 122/2)

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CRediT authorship contribution statement

Fernando A Gómez: Methodology, Formal analysis. **Francisca Villarroel:** Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis. **Nikol Ponce:** Writing – original draft, Methodology. **Francisco Nualart:** Writing – review & editing, Methodology, Formal analysis. **Paulo Salinas:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Methodology, Investigation, Formal analysis, Conceptualization. **Cristián Muñoz:** Methodology, Formal analysis. **Eder Ramírez:** Methodology, Formal analysis.

Declaration of Generative AI and AI-assisted technologies in the writing process

During the preparation of this work, the authors used ChatGPT to review and edit the correct translation from the original language to English. After using this tool, the authors reviewed and edited the content as necessary and take full responsibility for the content of the publication.

Declaration of Competing Interest

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

Data availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.reprotox.2024.108610.

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