

# Disrupted brain angiogenesis and blood–brain barrier function underlie cognitive deficits in offspring of preeclampsia-like pregnancies

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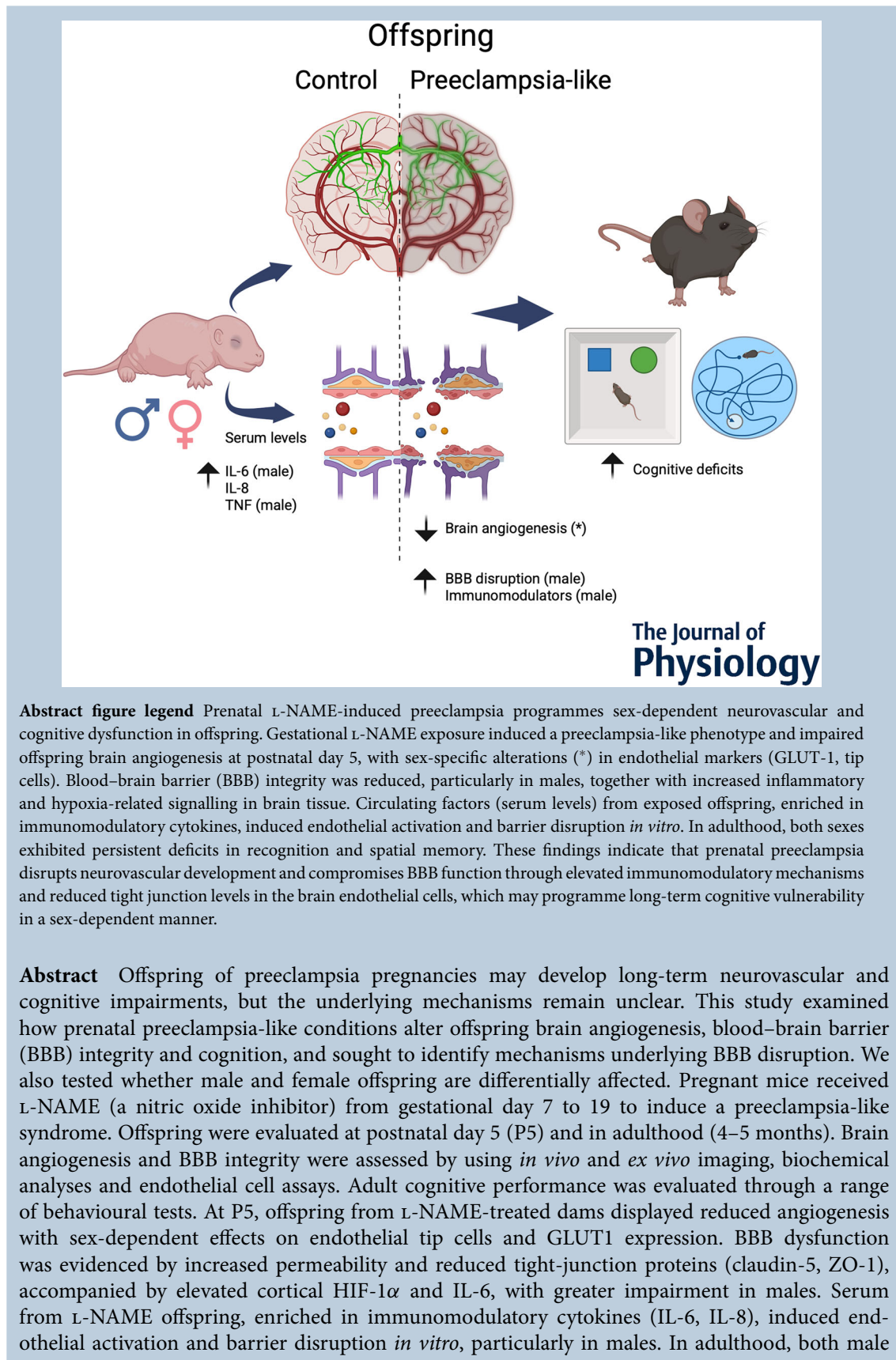
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**Felipe Andrés Troncoso Basso** holds a PhD in Veterinary Sciences from the University of Concepción (Chile) and is a postdoctoral researcher at the Vascular Physiology Laboratory of the University of Bio-Bio (Chile). His research focuses on the study of the oestrogen hormone receptor GPER1 and the role of sexual dimorphism in cerebral angiogenesis under physiological conditions, as well as in pathologies such as preeclampsia and stroke. **Carlos Escudero** is Full Professor and leader of the Vascular Physiology Laboratory at the University of Bio Bio (Chile). His research centres on placenta–brain communication, with his group investigating how placental extracellular vesicles disrupt the blood–brain barrier and impair brain angiogenesis in both mothers and babies exposed to preeclamptic pregnancies. He also studies circulating extracellular vesicles in stroke patients as biomarkers for stroke outcomes and brain vascular function.



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and female offspring exhibited deficits in recognition and spatial memory. Gestational exposure to a preeclampsia-like environment impairs early brain angiogenesis, disrupts BBB integrity via inflammatory and junctional mechanisms, and results in persistent cognitive dysfunction, revealing significant sex-specific vulnerability relevant to long-term neurological risk.

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### Key points

- Children born from pregnancies complicated by preeclampsia may face an increased risk of long-term brain and cognitive problems, but the biological causes have been unclear.
- Using a mouse model, we show that preeclampsia-like conditions may disrupt early brain blood vessel development and damage the brain's protective barrier.
- These changes are linked to inflammation and loss of proteins that normally protect brain blood vessels.
- Male offspring tend to be more severely affected than females, revealing important biological differences in vulnerability.
- Understanding these mechanisms is critical for developing strategies to protect the brain early in life and reduce the risk of long-term neurological problems in children born to women with preeclampsia.

## Introduction

Preeclampsia is a severe hypertensive disorder of pregnancy characterized by new-onset hypertension after 20 weeks of gestation, and signs of maternal organ damage, including proteinuria and neurological involvement (ACOG Task Force on Hypertension in Pregnancy 2013, 2019; Duley, 2009; Fishel Bartal & Sibai, 2022). It poses significant risks to both the mother and the fetus. Infants born to preeclamptic pregnancies have an increased risk of perinatal morbidity and mortality (Razak et al., 2018; Villar et al., 2006), with several studies suggesting male infants may be particularly vulnerable (Aliyu et al., 2012; Vatten & Skjaerven, 2004).

Beyond immediate perinatal outcomes, epidemiological studies indicate that exposure to maternal preeclampsia has an increased risk of long-term cardiovascular alterations in the offspring, including elevated blood pressure, increased relative wall thickness and reduced left ventricular end-diastolic volume, features that may help explain the heightened cardiovascular risk reported in individuals born to preeclamptic pregnancies (Timpka et al., 2016; Vatten et al., 2003). Experimental models of preeclampsia-like conditions parallel these findings and consistently show sex-dependent cardiovascular effects, with males exhibiting greater susceptibility than females (Beckers et al., 2021; Lu et al.,

2007), including accelerated cardiac maturation observed exclusively in male fetuses (Chatterjee et al., 2025).

In addition to cardiovascular consequences, the fetal brain is also susceptible to adverse intrauterine conditions. For instance, magnetic resonance imaging studies show reduced corpus callosum development (a sensitive indicator of intrauterine brain development maturation) in growth-restricted fetuses from preeclamptic pregnancies, a change linked to adverse perinatal outcomes (Zheng et al., 2022). Children born to mothers with preeclampsia also exhibit a heightened risk for perinatal stroke (Lee et al., 2005; Li et al., 2017; Wu et al., 2004), neonatal encephalopathy (Badawi et al., 1998; Cole et al., 2017), and long-term cognitive impairments associated with altered brain anatomy, connectivity and perfusion (Figueiro-Filho et al., 2017; Mak et al., 2018; Ratsep, Hickman, et al., 2016; Ratsep, Paolozza, et al., 2016). Supporting these findings, our recent post-mortem analysis showed increased cerebrovascular vulnerability in fetuses and neonates exposed to maternal hypertension, including a higher incidence of subarachnoid haemorrhage (Gonzalez et al., 2025). Collectively, these observations underscore the importance of cerebrovascular integrity in early life for maintaining long-term brain health (Tam & Watts, 2010; Whiteus et al., 2014).

Sex differences in cerebral blood flow (CBF) have been reported in healthy neonates, with males exhibiting higher CBF in the parietal, frontal and temporal lobes (Lin et al., 2013). Understanding how pregnancy complications such as preeclampsia affect these physiological differences remains unclear. Evidence on CBF and vascular function in offspring of preeclamptic pregnancies remains scarce. Only one small case-control study reported lower CBF in children born to women with preeclampsia, but without examining sex differences (Ratsep, Paolozza, et al., 2016).

Sex-specific differences have emerged as a relevant aspect of preeclampsia-related outcomes in cerebrovascular function. While some studies report a higher incidence of brain injury in male offspring (Carver et al., 2014; Hofsink et al., 2023), both sexes appear to be affected. We previously found that neonates (postnatal day 5, P5) from a preeclampsia-like model characterized by reduced uterine perfusion pressure (RUPP) showed greater signs of brain oedema than their control counterparts. Notably, brain perfusion deficits were more pronounced in female RUPP offspring (Lara et al., 2022). These findings highlight the sexually dimorphic nature of cerebrovascular vulnerability following prenatal hypertensive exposure.

Animal models have been essential for understanding how preeclampsia may affect brain development in offspring. Thus, the L-NAME-induced preeclampsia-like model, based on nitric oxide synthase inhibition, mimics several features of human preeclampsia in rodents, including maternal hypertension, proteinuria and fetal growth restriction (Troncoso et al., 2023). This model has been widely used to study brain alterations in offspring of preeclampsia pregnancies. Those reports have revealed impaired neurogenesis, reduced brain angiogenesis and cognitive deficits (Liu et al., 2016; Tachibana et al., 2019; Troncoso et al., 2023; Zhu et al., 2016), as well as a differential brain metabolomic profile (Xu et al., 2025) in exposed offspring. More recently, Wu et al. (2025) have reported that L-NAME offspring exhibit long-term neurodevelopmental and behavioural impairments in a sex-dependent manner, with males displaying deficits in anxiety-like behaviour, cognition and social interactions. Notably, these alterations were associated with reduced oligodendrocyte maturation, which involves wrapping axons with central cell membranes to form myelin. Importantly, they also found that female offspring were largely unaffected. However, the functional status of cerebral blood vessels, specifically their blood–brain barrier (BBB) properties, remains poorly characterized in this model.

Therefore, we investigated how prenatal preeclampsia-like conditions (generated by L-NAME administration) alter offspring brain angiogenesis, BBB integrity and cognition, and sought to identify mechanisms underlying BBB disruption. We also

examined whether male and female offspring are differentially affected.

## Methods

### Ethical approval

All procedures were approved by the Universidad del Bío-Bío Bioethics and Biosafety Committee and conducted in accordance with institutional guidelines, the *Guide for the Care and Use of Laboratory Animals* and the 3Rs principle (NIH 2011), under approval protocol Fondecyt 1240295. The experimental design and reporting adhere to ARRIVE 2.0 guidelines. C57BL/6 mice from in-house breeding colonies were maintained under controlled temperature (25°C) and humidity, and a 12:12 h light–dark cycle, with free access to food (Prolab RMH 3000, Labdiet, St. Louis, MO, USA) and water, as previously described (Cumsille et al., 2022; Lara et al., 2022).

### Preeclampsia-like models and experimental sample size

Female C57BL/6 mice (3–5 months old) were time-mated with age-matched males, and the presence of a vaginal plug was designated gestational day 0 (D0). A preeclampsia-like model was induced by administering the nitric oxide synthase inhibitor L-NAME (NG-nitroarginine methyl ester hydrochloride; 150 mg/kg/day in drinking water), from D7 to D19 of gestation, as previously reported (Burke et al., 2016) and validated by our group (Troncoso et al., 2023). On D19, a subset of pregnant mice were killed for standard characterization of the model (Burke et al., 2016), including systemic blood pressure, proteinuria, placental morphology, fetal morphometry, and circulating sFlt-1 and PlGF levels. A separate cohort of dams was allowed to deliver naturally to generate postnatal offspring for subsequent analyses.

Two developmental time points were analysed: postnatal day 5 (P5) and adulthood (4–5 months; see below). P5 corresponds to a peak phase of postnatal cerebrovascular growth, characterized by active angiogenesis, tip-cell expansion and maturation of the neurovascular unit (Harb et al., 2013; Menard et al., 2017). This period also represents a critical window for BBB tightening, during which junctional complexes, transporter systems and endothelial signalling pathways undergo rapid refinement (Engelhardt & Liebner, 2014; Obermeier et al., 2013). In contrast, the 4–5 month time point corresponds to young adulthood in mice, a period during which neurovascular function is stable and cognitive performance can be reliably evaluated without

confounding age-related vascular decline (Bennett et al., 2024).

Twenty dams (F0) were randomized into either the L-NAME ( $n = 10$ ) or the control group ( $n = 10$ ), with five dams per group evaluated at each postpartum time point. From these litters, 65 offspring (F1) were included: 35 controls (P5:  $n = 23$ ; adulthood:  $n = 12$ ) and 30 L-NAME (P5:  $n = 19$ ; adulthood:  $n = 11$ ). A complete characterization of the F1 preeclampsia-like model has been reported previously (Troncoso et al., 2023). In all experiments, one male and one female pup per litter were randomly selected and analysed as independent individuals. All experiments were performed in parallel, with matched control and L-NAME offspring processed simultaneously.

P5 pups and adult animals were killed by isoflurane overdose followed by rapid decapitation (P5) or cervical dislocation (adults), in accordance with institutional guidelines and the American Veterinary Medical Association (AWVMA, 2020). Immediately after decapitation, the skull was carefully opened using fine surgical scissors, and the intact brain was gently lifted out with micro-spatulas to avoid compression or mechanical damage. All dissections were carried out promptly to preserve tissue integrity and minimize post-mortem delay. For serum extraction, pups at P5 were killed, and blood was collected by cardiac puncture. Serum was separated by centrifugation (3000 rpm for 10 min) and stored at  $-80^{\circ}\text{C}$  for subsequent analysis.

### Adult animals

Offspring were weaned at day 21 postpartum and left until adulthood (4–5 months) under standard conditions ( $22^{\circ}\text{C}$ , 55% humidity and a 12 h light–dark cycle), with free access to food and water throughout the entire period. Animal care followed the directives approved by the Ethics and Animal Care Committee of the Universidad de Valparaíso (BEA160-20) and the Universidad del Biobío.

### Cortex brain blood vessel immunohistochemistry

The motor and somatosensory cortices were selected for evaluating brain blood vessels with and without functional lumens, as reported previously (Troncoso et al., 2023). At P5, pups were placed under light isoflurane anaesthesia (1–2% in oxygen) prior to retro-orbital injection of vascular-selective lectin (Wheat Germ Agglutinin, Alexa Fluor 488 conjugate; Thermo Fisher, Waltham, MA, USA). Injections were performed using a 30G needle (10  $\mu\text{L}$  total volume; 50  $\mu\text{g}/\text{mL}$ ), and blanching of the retro-orbital sinus was used to confirm intravenous delivery. Following labelling, pups were killed as described above, and brains

were fixed in 4% paraformaldehyde for 24 h. Coronal sections (200  $\mu\text{m}$ ) were obtained using a vibratome.

Lectin-labelled cortical vessels were imaged using a confocal microscope (LSM780, Zeiss, Oberkochen, Germany). Vascular density, total vessel length and number of junctions were quantified using AngioTool, after conversion to 16-bit greyscale and segmentation/skeletonization.

In an independent cohort (P5;  $n = 5$  per group), endothelial tip cells were quantified in 100  $\mu\text{m}$  cortical sections immunostained for CD31 (1:300; AF3628, R&D Systems, Minneapolis, MN, USA) and visualized with Alexa Fluor 594-conjugated secondary antibodies. Images were acquired at  $40\times$  magnification (Zeiss LSM800), and tip cells were identified morphologically by a blinded investigator. Counts were normalized to the total CD31<sup>+</sup> area.

### Brain homogenate preparation

P5 pups were killed as indicated above, and their brains were quickly removed and placed on ice. A 3 mm cortical section adjacent to the hippocampus was homogenized in 200  $\mu\text{L}$  lysis buffer containing protease inhibitors using a WiseTis homogenizer (500 rpm, 1 min). The homogenate was centrifuged at 14,000 rpm for 10 min at  $4^{\circ}\text{C}$ , and the supernatant was collected for protein quantification using the Pierce BCA Protein Assay Kit (Thermo Fisher, Rockford, IL, USA #23 227). Subsequently, the samples were stored at  $-80^{\circ}\text{C}$ . For protein expression experiments, we used a protein concentration of 50  $\mu\text{g}$ .

### Primary fetal mice brain endothelial cells

Brain microvascular endothelial cells were isolated from wild-type C57BL/6 pups at embryonic day 18.5 (E18.5) using a modified protocol (Ouellette & Lacoste, 2021). Briefly, following cervical dislocation and immediately after decapitation, the skull was opened with fine scissors, and the brain was gently removed with micro-spatulas to avoid mechanical damage. All dissections were performed rapidly to preserve tissue integrity and minimize post-mortem delay. The pup's brains were removed, and the meninges, cerebellum and brainstem were discarded, leaving the cerebral hemispheres. The tissue was dissociated with collagenase (1 mg/mL) and DNase (1 mg/mL) for 2 h at  $37^{\circ}\text{C}$ . The cell suspension was centrifuged, and the pellet was resuspended in bovine serum albumin (BSA)-DMEM (20% w/v), followed by a second centrifugation. Cells were cultured in DMEM-EndoGro<sup>TM</sup> medium (Millipore, Billerica, MA, USA) at  $37^{\circ}\text{C}$  and 5%  $\text{CO}_2$ . Endothelial cells were immunoselected using Dynabeads

CD31 (Invitrogen, Vilnius, Latvia) according to the manufacturer's instructions (Troncoso et al., 2017).

### Bend3 cell culture

Bend3 cells (brain microvascular endothelial cells derived from BALB/c mice) were cultured as previously described (Sandoval et al., 2024). Briefly, cells were maintained in Dulbecco's modified Eagle Medium (DMEM, high glucose, Invitrogen), supplemented with 10% fetal bovine serum (FBS, Thermo Fisher Scientific), and 1% penicillin/streptomycin (Gibco) at 37°C in a humidified atmosphere with 5% CO<sub>2</sub>. To differentiate toward a more endothelial-like phenotype, cells were grown to confluence and then treated with 20% fetal bovine serum for 24 h. The culture medium was replaced regularly, and cells were passaged when they reached 80–90% confluence. Experiments were performed between passages 5 and 10.

### In vitro angiogenesis

Bend3 cells ( $4 \times 10^4$ ) were seeded onto 96-well plates pre-coated with 40 µL of Matrigel basement membrane matrix (Corning Labware, NY, USA). Cells were treated with serum from P5 pups (1% v/v) for 6 h, as previously described (Sandoval et al., 2024). Tube formation was observed using an inverted phase-contrast microscope (10× magnification, Olympus, Tokyo, Japan). Network formation, including branch points and junctions, was quantified using the 'Angiogenesis Analyzer' plugin in ImageJ 1.48, as outlined in prior studies (Troncoso et al., 2017, 2020).

### Measurement of transendothelial electrical resistance and cell permeability

Transendothelial electrical resistance (TEER) and FITC-dextran (70 kDa) permeability were measured as previously described (Bergman et al., 2021). TEER was assessed using an EVOM2 volt-ohmmeter (World Precision Instruments, Sarasota, FL, USA) on transwell plates with inserts (Corning, Glendale, AZ, USA #3401) cultured with Bend3 cells and/or fetal mice brain endothelial cells (fmBECs), as specified in the experimental assay. An initial resistance of approximately 200 Ω cm<sup>2</sup> ( $T = 0$ ) was considered for each experimental condition, and a second reading was subsequently taken after 12 h of incubation ( $T = 12$ ) with serum (50 µg/mL) from P5 pups. Data from TEER measurements were expressed as percentage change according to the formula:  $(Teer_{12h} - Teer_{0h}) \times 100 / Teer_{0h}$ .

For permeability assays, 50 µL FITC-dextran (1 µM) was applied to these inserts for 30 min at 37°C. Baso-

lateral medium was collected for fluorescence analysis using a Synergy HTX 2 plate reader (BioTek Instruments, Winooski, VT, USA), with excitation and emission wavelengths of 485 and 540 nm, respectively, and background values were subtracted from cell-free wells.

### In vivo FITC-dextran permeability assay

BBB permeability *in vivo* was assessed by retro-orbital injection of 70 kDa FITC-dextran (50 µg/g body weight) into lightly isoflurane-anaesthetized P5 pups. After 10 min, pups were killed and perfused with 1 mL warm PBS. Cortex overlying the hippocampus was dissected, homogenized and centrifuged (2000 rpm, 10 min), and supernatants were analysed using a Synergy HTX2 spectrophotometer (excitation 488 nm/emission 534 nm). Fluorescence was normalized to total protein.

### Immunocytochemistry, KDR, CD31 and claudin 5

Bend3 cells were treated with serum from P5 offspring (50 µg/mL) or vehicle control for 12 h. Cells were fixed in 70% ethanol diluted in PBS (30 min, 4°C) and blocked with 5% BSA for 20 min. Primary antibodies were applied overnight at 4°C: anti-claudin-5 (1:250; Santa Cruz sc-374 221; Santa Cruz, CA, USA), anti-KDR/VEGFR2 (Cell Signaling Technology, Danvers, MA, USA) and anti-CD31 (1:250; Abcam ab9498; Cambridge, MA, USA). After washing, cells were incubated with the appropriate Alexa Fluor-conjugated secondary antibodies (Alexa 594 anti-mouse IgG, Alexa 488 anti-rabbit IgG; 1:500; Thermo Fisher). Nuclei were counterstained with DAPI (1:1000; Sigma, St Louis, MO, USA).

Fluorescence images were acquired using a Jenoptik ProgRes SpeedXT camera at 100× magnification. For each condition, three random fields were imaged, and fluorescence intensity was quantified using ImageJ v1.48 (Fitzpatrick, 2014).

### Immunoblotting of brain, serum and cells

Western blotting was performed on brain homogenates, serum and Bend3 cells. Brain tissues were homogenized (as described above), while serum was collected via retro-orbital puncture and stored at -80°C. At the same time, Bend3 cells were stimulated with 50 µg/mL serum from P5 mice (control or L-NAME offspring) for 12 h, followed by lysis and centrifugation. Protein concentrations were then quantified using the BCA assay. SDS-PAGE was performed on 10% acrylamide gels, using 50 µg of protein per sample. Proteins were transferred to nitrocellulose membranes, stained with Ponceau Red and incubated overnight with primary antibodies.

The following antibodies were used, depending on the biological sample being analysed. For brain homogenates: anti-CD31 (Abcam), anti-CLDN5, anti-IL-6, anti-TNF- $\alpha$ , anti-albumin, anti-HIF-1 $\alpha$  (Santa Cruz Biotechnology, Dallas, TX, USA) and anti-GLUT-1 (Sigma-Aldrich). For serum, anti-IL-8, anti-IL-6 and anti-TNF- $\alpha$  (Santa Cruz Biotechnology) were used. For Bend3 cells, antibodies against ZO-1, CD31, CLDN5, IL-6, IL-8 and TNF- $\alpha$  (all from Santa Cruz Biotechnology) were applied. After HRP-conjugated anti-mouse IgG (Sigma-Aldrich) incubation, chemiluminescence signals were detected using a GeneGnome XRQ system (Syngene, Bangalore, India). Densitometric analysis was performed using ImageJ, with protein expression normalized to  $\beta$ -actin (Santa Cruz Biotechnology).

### Real-time quantitative PCR

Real-time quantitative PCR (qRT-PCR) was performed using the SYBR Green/ROX master mix (Thermo Fisher Scientific) on an Mx3000P QPCR system (Agilent Technologies, Santa Clara, CA, USA). Reactions (20  $\mu$ L) were run with an initial denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 60°C for 15 s and 72°C for 20 s. Reactions were performed in duplicate, and data were analysed using the 2<sup>dd</sup>Ct method, with GAPDH as the reference gene.

We used the following primers:

IL-6:

Forward 5'-CCAACAGACCTGTCTATACC-3';

Reverse 5'-CTGCAAGTGCATCATCGTTG-3'

TNF- $\alpha$ :

Forward 5'-AAAGCATGATCCGCGACGTG-3';

Reverse 5'-CAGGAATGAGAGGCTGAG-3'

CLDN5:

Forward 5'-ATTCTGGGTCTGGTGCTGTG-3';

Reverse 5'-CACGATGTTGTGGTCCAGGA-3'

CD31:

Forward 5'-AGGAAAGCCAAGGCCAAA-3';

Reverse 5'-TTGACTGTCTTAAGTTCC-3'

GAPDH:

Forward 5'-TTGCCATCAACGACCCCTTC-3';

Reverse 5'-GGGTCTCGCTCCTGGAAAATC-3'

### Open field test and novel object recognition test

The open field test (OFT) and novel object recognition test (NORT) were performed in a 40  $\times$  40 cm white acrylic arena. For the OFT, mice were placed in the centre and allowed to explore freely for 5 min. Total distance travelled and locomotor speed were recorded.

The NORT was conducted over 2 days. On day 1 (familiarization), mice explored two identical objects for 5 min, and the time spent exploring each object was

recorded. On day 2 (test phase), one object was replaced with a novel object, and mice were allowed to explore for 5 min. Exploration was scored when the mouse's nose or forepaws contacted or oriented toward the object. Preference for the novel object was calculated as the percentage of time spent exploring the novel *versus* the familiar object.

### Place recognition test

The place recognition test (PRT) was conducted in a 50  $\times$  40  $\times$  63 cm acrylic box over three consecutive days. On day 1 (habituation), animals were placed in the centre, facing a cue (a bold 'A') on the north wall, and allowed to explore the empty box for 5 min. On day 2 (acquisition phase), two identical objects were placed in the centre, and the exploration time for each object was recorded. On day 3 (choice phase), one object was moved to a new location, introducing novelty. The time spent exploring the familiar and displaced objects was quantified as a percentage of total exploration time. The box and objects were cleaned with alcohol between animals to avoid odour cues. This hippocampus-dependent task tests spatial memory, as mice must recall the relative positioning of two similar objects.

### Morris water maze test

The Morris water maze test (MWM) was conducted in a circular pool (120 cm in diameter, 60 cm in height, 40 cm in water depth) surrounded by visual cues. Mice were habituated on day 1 by swimming for 60 s. On day 2, mice were trained in three trials to locate a visible platform elevated 1 cm above the water surface. For the next 4 days, a hidden platform was submerged (1 cm) below the water surface, and mice were trained to find it using visual cues (acquisition phase), with six trials per day at 15 min intervals. On day 5, 24 h after the last acquisition trial, a probe trial was conducted to assess spatial memory. Latency, number of platform crossings and time spent in the target quadrant were measured. Behavioural data were analysed using Anymaze software (v.7.09, Stoelting Co., Wood Dale, IL, USA).

### Statistical analysis

Quantitative variables are presented as median  $\pm$  SD, whereas qualitative variables are presented as percentages within each group. Considering data distribution, we employed parametric or non-parametric tests, as appropriate, based on the results of the Shapiro–Wilk normality test. Then, we compared the groups using a Student's *t* test or a Mann–Whitney test. In analyses of brain homogenates and *in vitro* studies conducted

**Table 1. Characterization of P5 offspring of L-NAME dams**

Characteristic	Control male D19 (n=19)	L-NAME male D19 (n=18)	Control female D19 (n=17)	L-NAME female D19 (n=20)	Two-way ANOVA
Litter size, $n \pm SD$	3.8 $\pm$ 1.9	3.4 $\pm$ 1.6	3.6 $\pm$ 2.0	4.0 $\pm$ 1.2	0.72
Weight D19, g	1.1 $\pm$ 0.1	1.1 $\pm$ 0.1	1.1 $\pm$ 0.1	1.0 $\pm$ 0.1	0.05
Length D19, mm	21.3 $\pm$ 1.4	21.7 $\pm$ 1.2	20.2 $\pm$ 1.7	19.4 $\pm$ 2.5*	0.05
Width D19, mm	11.8 $\pm$ 0.8	11.5 $\pm$ 1.1	11.1 $\pm$ 1.4	10.4 $\pm$ 1.7*	0.05
	Control male P5 (n=16)	L-NAME male P5 (n=7)	Control female P5 (n=7)	L-NAME female P5 (n=12)	
Weight P5, g $\pm$ SD	3.0 $\pm$ 0.4	2.0 $\pm$ 0.2*	2.7 $\pm$ 0.4	2.1 $\pm$ 0.3*	0.05
Length P5, mm $\pm$ SD	36.2 $\pm$ 2.1	29.4 $\pm$ 1.9*	35.7 $\pm$ 1.2	31.0 $\pm$ 2.7*	0.05
Width, mm $\pm$ SD	12.0 $\pm$ 0.6	10.1 $\pm$ 0.7*	11.9 $\pm$ 0.6	10.7 $\pm$ 0.6*	0.05

\*  $P < 0.05$  versus respective control group.

head-to-head, a paired  $t$  test was used. When both sexes were evaluated for the same experimental outcome, we used a two-way ANOVA with sex and treatment as factors to assess main effects and potential interactions; when no significant interaction was detected, we reported overall treatment effects, with sex-stratified values presented descriptively. Because most experimental measures were obtained from different subsets of animals, correlation analyses were not conducted to avoid biased or unreliable associations based on limited sample overlap.  $P < 0.05$  was considered statistically significant. Data and statistical analyses were performed using a Microsoft Excel database and GraphPad Prism 6 (GraphPad Software, La Jolla, CA, USA).

## Results

### Offspring from preeclampsia-like syndrome showed reduced brain angiogenesis

Our group has previously characterized the preeclampsia-like syndrome induced by L-NAME administration (Troncoso et al., 2023).

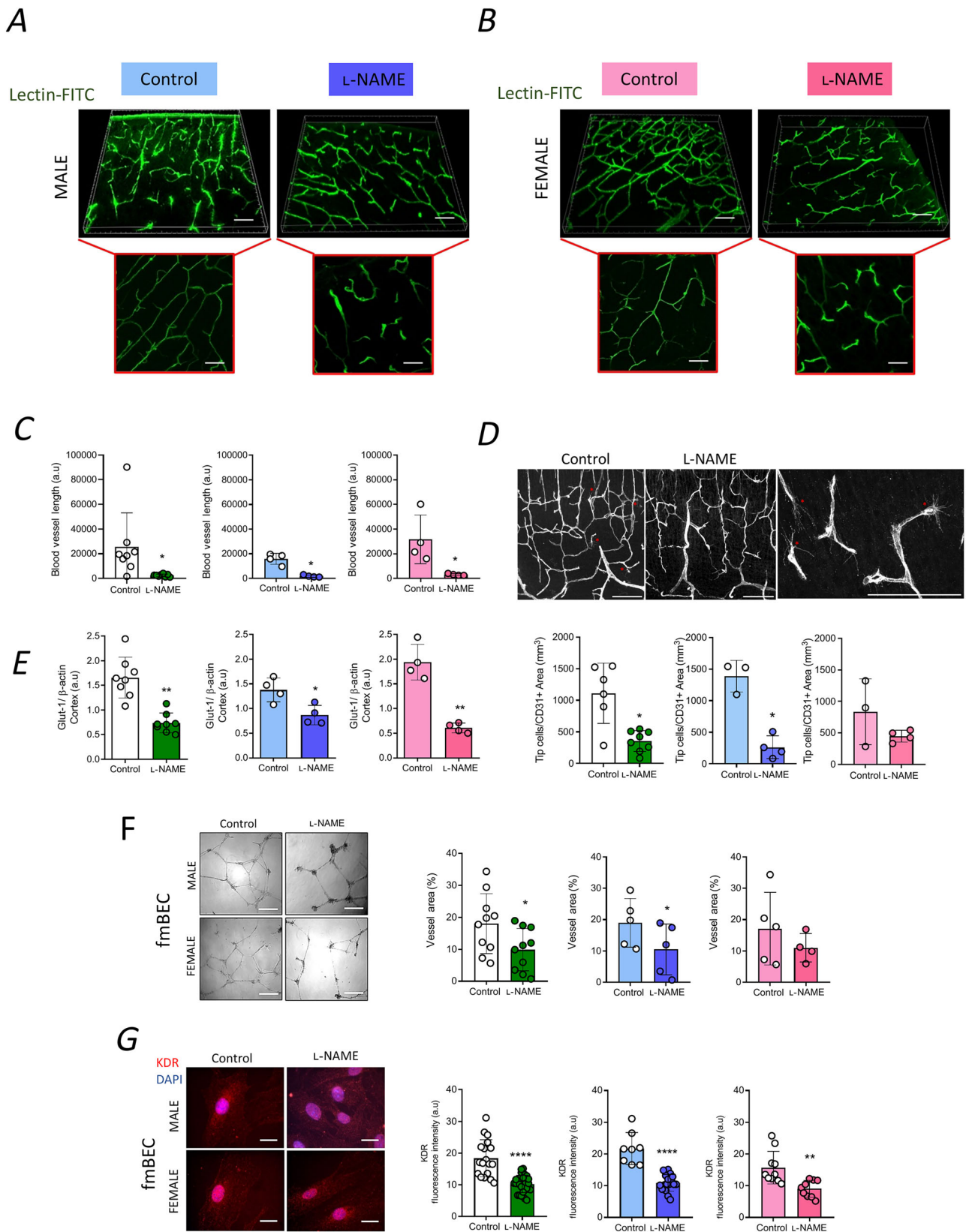
At gestational day 19, two-way ANOVA identified a significant main effect of treatment on overall pup size ( $P = 0.05$ ), indicating that offspring from L-NAME pregnancies were smaller than controls when analysed as a group (1.05  $\pm$  0.12 vs. 1.10  $\pm$  0.09 g). No significant sex  $\times$  treatment interaction was observed. *Post hoc* comparisons showed that the reduction in fetal size was primarily attributable to females, as female L-NAME fetuses exhibited shorter crown-rump length and reduced body width compared with female controls (*post hoc*  $P < 0.05$ ). In contrast, male L-NAME and control fetuses did not differ (Table 1).

At P5, offspring from L-NAME-treated dams remained smaller than controls (Table 1). Consistent with the fetal

findings, two-way ANOVA again revealed no significant sex  $\times$  treatment interaction for any anthropometric measure, indicating that the postnatal growth reduction reflected a general treatment effect rather than a sex-specific response.

We previously showed reduced brain angiogenesis in offspring from L-NAME dams at P5 (Troncoso et al., 2023). Here, to evaluate whether prenatal L-NAME exposure produced sex-dependent alterations in early brain angiogenesis, we quantified two complementary parameters: (i) endothelial tip cell density (Walchli et al., 2015) and (ii) GLUT1 protein levels as a marker of brain endothelial content (Nualart et al., 1999). Two-way ANOVA revealed a significant sex  $\times$  treatment interaction for both measures [tip cells:  $F(1,10) = 5.85$ ,  $P = 0.036$ ; GLUT1:  $F(1,12) = 11.51$ ,  $P = 0.0053$ ] alongside a strong main effect of treatment ( $P < 0.0001$ ). Although baseline values differed between male and female controls, the direction and magnitude of the response to L-NAME diverged by sex, consistent with a sex-specific vulnerability captured by the interaction term. For all other angiogenic, vascular and BBB-related parameters, two-way ANOVA did not show significant sex  $\times$  treatment interactions. In these cases, we therefore report (i) pooled analyses to estimate overall treatment effects and (ii) sex-stratified comparisons when relevant to describe sex-group differences not supported by interaction effects.

Compared to controls, offspring from L-NAME dams exhibited significantly lower vascular density (Fig. 1A–C), shorter vessel length (data not shown), decreased expression of GLUT1 (Fig. 1E) and fewer tip endothelial cells (Fig. 1D) in the brain cortex. These reductions were evident in both sexes from L-NAME dams (Fig. 1A–E). However, only male offspring from L-NAME dams displayed significantly lower tip cells than male controls (Fig. 1D).



quantification of vessel length in male (blue bars) and female (pink bars) P5 pups. *D*, representative images of cortical vasculature in pups from control and L-NAME dams, with red dots indicating identified endothelial tip cells. The graph below shows quantification of tip cells per area across the analysed groups. *E*, densitometric analysis of GLUT-1 (brain endothelial marker) normalized to  $\beta$ -actin in cortical homogenates from male and female P5 pups. *F*, representative images of fetal brain endothelial cells (fmBECs) treated with serum from male and female P5 pups. Graphs indicate quantification of *in vitro* angiogenic capacity as vessel area in comparative groups. *G*, representative immunofluorescence images of KDR (VEGFR2) and its quantification in fmBECs exposed to serum from male and female P5 pups. Each dot represents one pup (one male and one female per litter). Data are presented as median  $\pm$  SD. \* $P < 0.05$ , \*\* $P < 0.005$ , \*\*\*\* $P < 0.0001$  versus respective control group.

Given previous reports of dysregulated circulating factors impairing endothelial angiogenic capacity in offspring exposed to preeclampsia (Kvehaugen et al., 2011; Troncoso et al., 2023), we confirmed this effect in fmBEC cells treated with serum (50  $\mu$ g/mL, 3 h) from P5 offspring of L-NAME-treated dams (Fig. 1F). Further analysis revealed that serum from male – but not female – offspring from L-NAME-treated dams significantly reduced angiogenic capacity in fmBECs, as determined by reduction in vessel area (Fig. 1F) and the number of vessel junctions (data not shown).

Furthermore, serum from L-NAME offspring significantly reduced KDR/VEGFR2 protein levels (the critical proangiogenic receptor) in fmBECs (Fig. 1G), with similar reductions observed in both male- and female-derived cells (Fig. 1G). Together, these results indicate that prenatal exposure to preeclampsia-like conditions impairs proangiogenic signalling, with a probably more pronounced effect in male offspring.

### Offspring from L-NAME showed an impaired BBB

A key functional property of brain microvessels is the formation of the BBB. To assess BBB integrity, we examined cortical endothelial markers. Offspring from L-NAME dams showed increased CD31 (PECAM-1) expression compared with controls (Fig. 2A and B), consistent with endothelial activation. This increase reached statistical significance only in males (Fig. 2B).

In contrast, expression of CLDN5, a tight junction protein and key determinant of BBB integrity (Greene et al., 2019), was significantly reduced at both the mRNA and protein levels in offspring from L-NAME dams compared to controls (Fig. 2C and D). This reduction was observed in both sexes (Fig. 2C and D), although it did not reach statistical significance in females.

To evaluate BBB function directly, we measured *in vivo* FITC–dextran (70 kDa) extravasation (Fig. 2E). L-NAME offspring exhibited significantly greater cortical FITC–dextran accumulation than controls (Fig. 2F), consistent with increased BBB permeability. This increase was more pronounced in males, in which significance was retained after sex stratification (Fig. 2G).

We next quantified cortical albumin, a plasma protein typically excluded from the brain parenchyma by an

intact BBB. Albumin levels were significantly elevated in L-NAME offspring (Fig. 2H and I), confirming increased permeability. Sex-stratified analysis showed that this elevation was predominantly driven by males (Fig. 2J).

### Brain hypoxic and inflammatory response in offspring from L-NAME dams

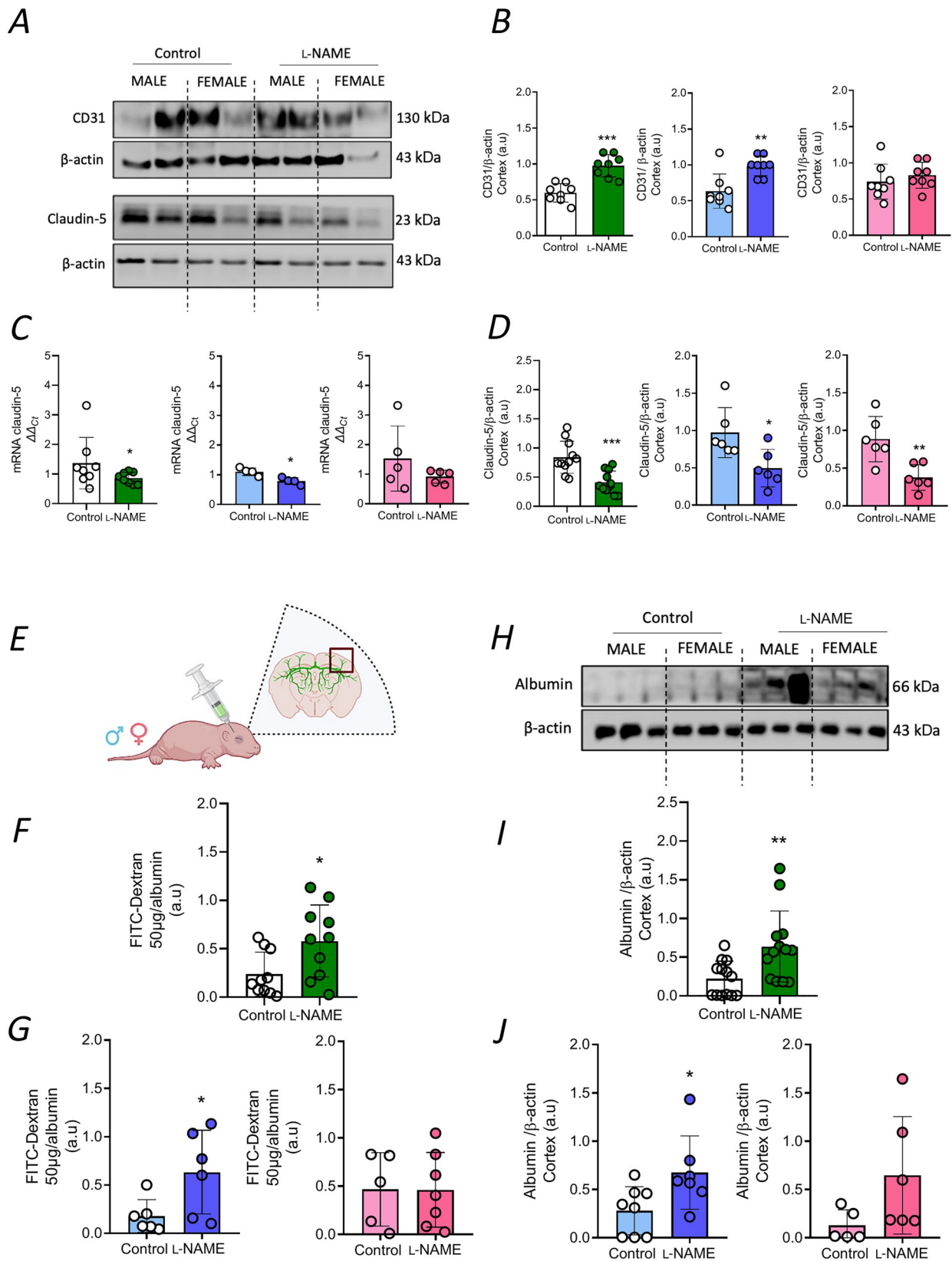
To determine whether cerebrovascular impairment was associated with hypoxic or inflammatory signalling, we measured cortical levels of hypoxia-inducible factor 1 $\alpha$  (HIF-1 $\alpha$ ) and the immunomodulatory cytokines IL-6 and TNF- $\alpha$  (Fig. 3A). L-NAME offspring exhibited increased HIF-1 $\alpha$  protein (Fig. 3B) and elevated IL-6 expression at both the mRNA (Table 2) and protein levels (Fig. 3C). In contrast, TNF- $\alpha$  expression was reduced at both the mRNA and protein levels in L-NAME offspring (Table 2, Fig. 3D).

Sex-stratified analysis revealed that elevations in HIF-1 $\alpha$  and IL-6 protein levels remained statistically significant in male offspring of L-NAME-treated dams compared to their counterparts in the control group (Fig. 3B and C). However, in female L-NAME offspring, only IL-6 protein levels were significantly increased (Fig. 3C). We found no changes in the synthesis of TNF- $\alpha$  at both the mRNA and protein levels in male and female offspring between the L-NAME and control groups (Fig. 3D, Table 2).

### Immunomodulatory profile in the brain cortex

We next examined whether cortical cytokine alterations were reflected systemically by measuring the circulating immunomodulatory cytokines IL-6, IL-8 and TNF- $\alpha$  (Fig. 4A). L-NAME offspring showed elevated serum IL-6 and IL-8 levels compared with controls (Fig. 4B and C). Serum TNF- $\alpha$  levels were highly variable and did not differ significantly between controls and L-NAME groups (Fig. 4D).

Stratification by sex revealed that circulating levels of IL-6 were significantly elevated in male offspring from L-NAME treated dams. In contrast to the whole-group analysis, TNF- $\alpha$  levels were also significantly elevated in male offspring from L-NAME-treated dams compared with male controls (Fig. 4B and D). Nevertheless, the pre-



**Figure 2. Disruption of the blood–brain barrier in male and female offspring of preeclampsia pregnancies**

A, representative blots of the endothelial markers CD31 and CLDN5 in brain cortex homogenates from P5 pups of control (white bars) and L-NAME (green bars) dams. B, densitometric analysis of CD31 normalized to β-actin

in cortical homogenates. Male (blue bars) and female (pink bars) P5 pups. *C* and *D*, mRNA (*C*) and protein (*D*) levels of the tight junction protein Claudin 5 in cortical homogenates from male and female P5 pups. *E*, schematic of the experimental approach with intraocular injection of FITC–dextran (70 kDa). The cartoon was created with BioRender. *F*, graphs indicating quantification of FITC–dextran (70 kDa) extravasation in cortical homogenates from all subjects, with separate analyses in (*G*) male (blue bars) and female (pink bars) pups. *H*, representative blot of albumin extravasation in brain cortex homogenates. *I*, densitometry of albumin normalized to  $\beta$ -actin in cortical homogenates from control and L-NAME pups, or (*J*) separated analysis of male and female P5 pups. Each dot represents one pup (one male and one female per litter). Data are presented as median  $\pm$  SD. \* $P < 0.05$ , \*\* $P < 0.005$  versus respective control group.

**Table 2. IL-6 and TNF- $\alpha$  mRNA levels in brain cortex homogenates of P5 pups from L-NAME dams**

Gene	Control ( $n=8$ )	L-NAME ( $n=8$ )	Control female ( $n=4$ )	L-NAME female ( $n=4$ )	Control male ( $n=4$ )	L-NAME male ( $n=4$ )
IL-6 $\Delta\Delta$ CT	1.1 $\pm$ 0.4	1.9 $\pm$ 1.1*	1.2 $\pm$ 0.6	1.6 $\pm$ 0.8	0.9 $\pm$ 0.3	2.2 $\pm$ 1.5
TNF- $\alpha$ $\Delta\Delta$ CT	0.7 $\pm$ 0.3	0.3 $\pm$ 0.2*	0.6 $\pm$ 0.3	0.3 $\pm$ 0.3	0.8 $\pm$ 0.3	0.4 $\pm$ 0.2

\* $P < 0.05$  versus control; † test.

viously observed differences in IL-8 levels were no longer significant following sex-specific analysis (Fig. 4C). These findings suggest a partial overlap between systemic and cerebral inflammatory responses, with more pronounced systemic changes observed in male offspring.

### Serum from offspring of L-NAME dams triggered an immunomodulatory response in brain endothelial cells

To assess whether circulating factors from L-NAME-exposed offspring could activate brain endothelial cells, we treated Bend3 cells with serum from P5 pups (Fig. 4A). Compared to control serum, serum from L-NAME-treated offspring induced significantly higher protein levels of IL-6 (Fig. 4E), IL-8 (Fig. 4F), TNF- $\alpha$  (Fig. 4G) and the endothelial activation marker CD31 (data not shown).

Sex-stratified analyses showed that serum from both male and female L-NAME offspring significantly increased IL-6 and TNF- $\alpha$  synthesis in Bend3 cells (Fig. 4E and G). In contrast, IL-8 was significantly induced only by serum from male L-NAME offspring (Fig. 4F). The increase in CD31 observed in pooled analyses did not remain significant after sex stratification (data not shown). These findings indicate that circulating factors from L-NAME offspring can activate brain endothelial cells, with specific immunomodulatory effects – particularly IL-8 induction – being more pronounced in response to male-derived serum.

### Serum from offspring of L-NAME dams impairs the BBB *in vitro*

We next tested whether circulating factors from L-NAME offspring directly impaired BBB properties *in vitro*.

Serum from L-NAME offspring significantly reduced TEER (Fig. 5A), increased permeability to FITC–dextran (Fig. 5B), and decreased expression of the tight-junction proteins claudin-5 (CLDN5; Fig. 5C and D) and zonula occludens-1 (ZO-1; Fig. 5E). Together, these findings indicate that serum from L-NAME offspring disrupts key structural and functional components of the endothelial barrier.

Sex-stratified analysis revealed that serum from male L-NAME offspring significantly impaired all BBB readouts – reducing TEER (Fig. 5A), increasing FITC–dextran permeability (Fig. 5B), and decreasing CLDN5 and ZO-1 expression (Fig. 5C–E). In contrast, serum from female L-NAME offspring produced a selective reduction in TEER (Fig. 5A) without significant changes in permeability or tight-junction protein levels. These results suggest that while circulating factors from both sexes can weaken BBB electrical resistance, serum from male L-NAME offspring exerts broader and more pronounced barrier-disrupting effects.

To validate these findings, we treated fmBECs with serum from L-NAME and control offspring (Fig. 6). Serum from offspring of L-NAME dams significantly increased CD31 protein expression, indicating endothelial activation (Fig. 6A and B). This effect was statistically significant only in cells treated with serum from male offspring (Fig. 6B).

Consistent with findings in the adult brain endothelial cell line Bend3 (Fig. 5), serum from L-NAME offspring impaired barrier function in fmBECs, as evidenced by decreased TEER, increased FITC–dextran permeability and reduced CLDN5 protein expression (Fig. 6C–F). Notably, barrier disruption remained significant in fmBECs treated with serum from male offspring, as shown by reduced TEER (Fig. 6C) and increased permeability (Fig. 6D). In contrast, CLDN5 expression was similarly

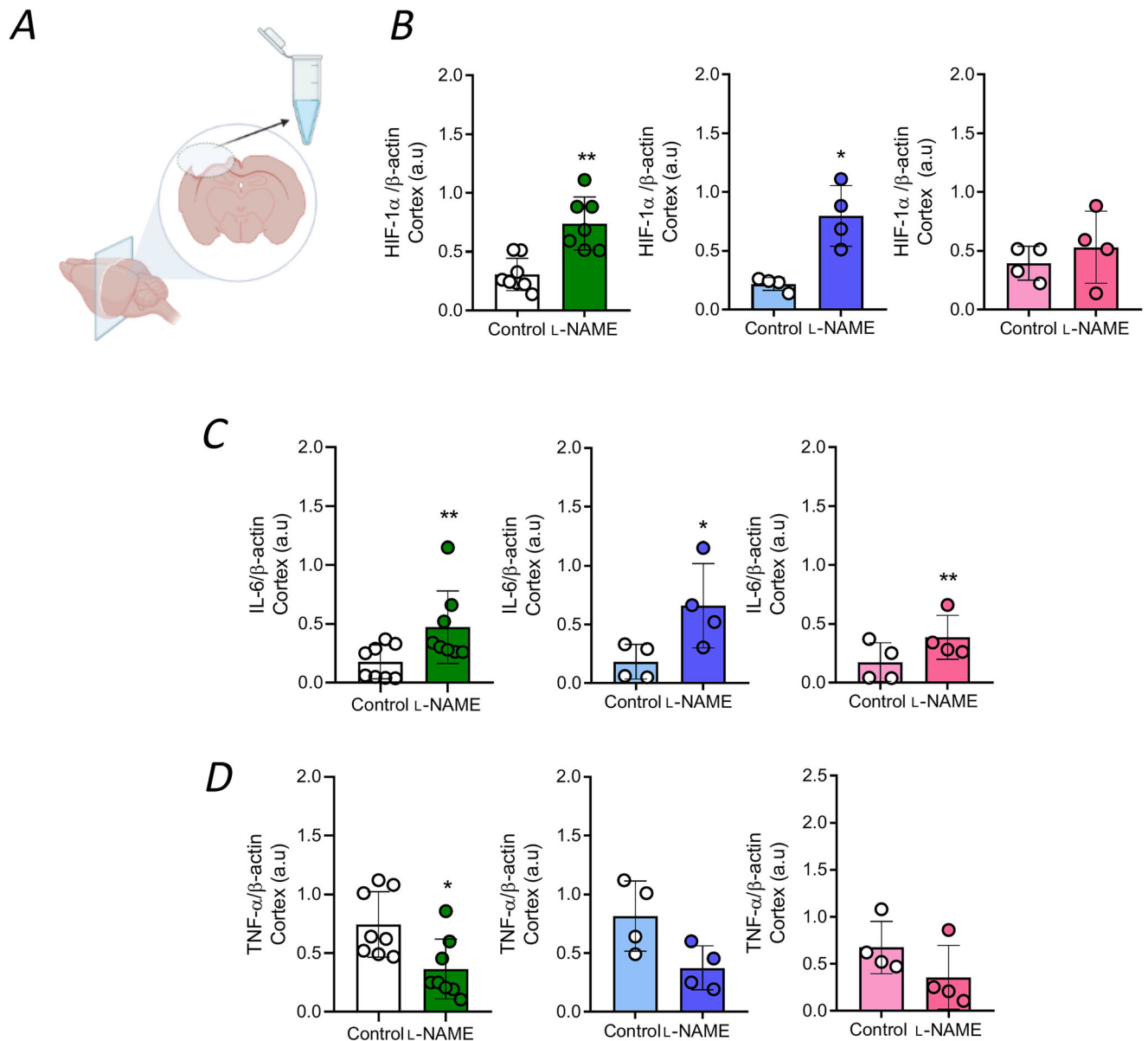
decreased following exposure to serum from both male and female offspring (Fig. 6E and F), suggesting that some aspects of barrier disruption are sex-independent.

### Adult consequences of impaired brain vascular function in offspring from L-NAME dams

To evaluate long-term consequences of brain vascular alterations, cognitive performance was assessed in

adult offspring from L-NAME-treated dams using four behavioural tests: OFT, NORT, PRT and MWMT (Fig. 7A). No significant differences were observed in total distance travelled (Fig. 7B) or locomotor velocity (Fig. 7C) in the OFT, indicating comparable baseline motor activity across groups.

In the NORT and PRT, control offspring demonstrated a clear preference for the novel object or displaced location (Fig. 7D and E), reflecting intact recognition and



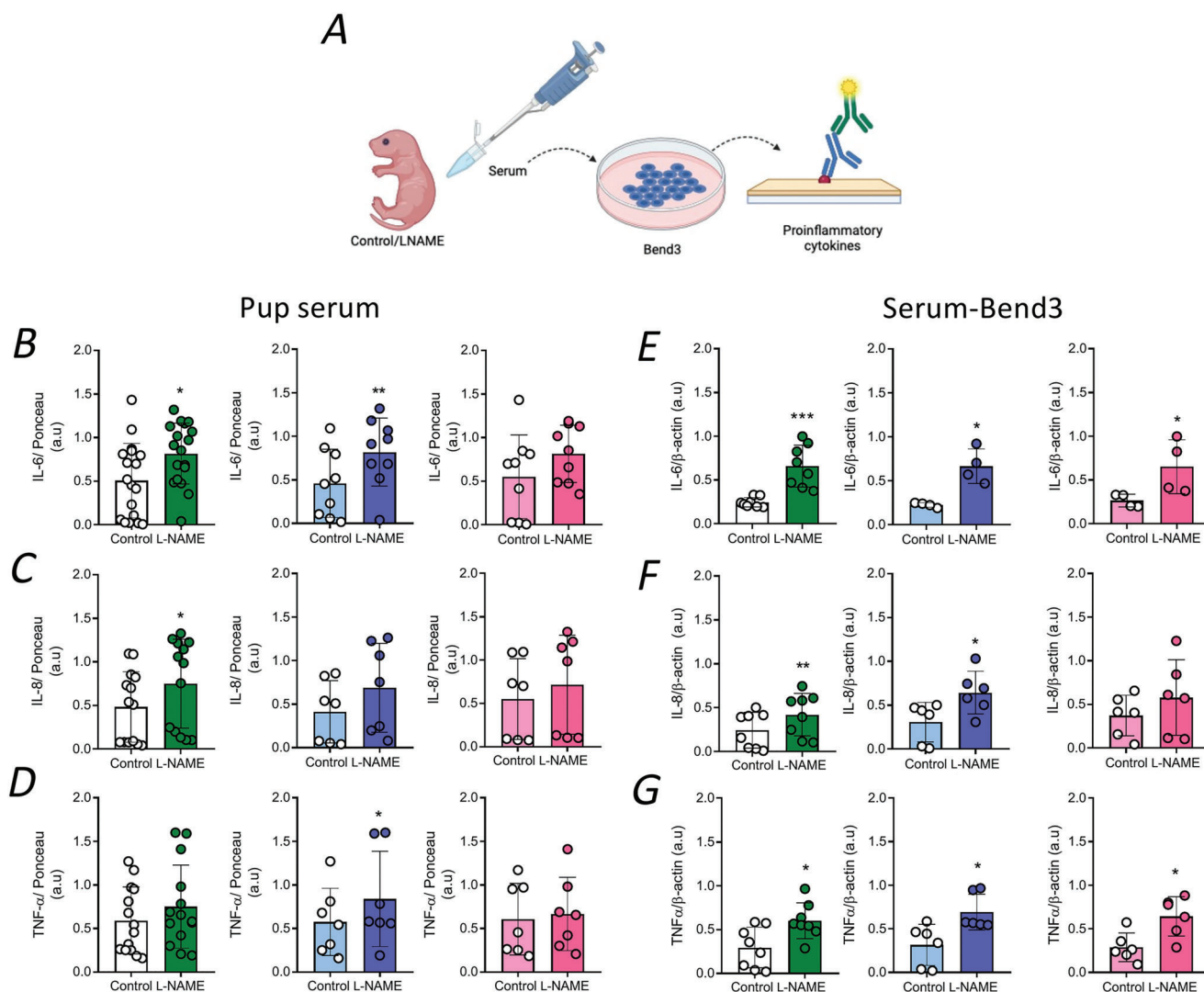
**Figure 3. Immunomodulatory profile in the brains of male and female offspring of mothers with preeclampsia**

A, schematic of brain cortex homogenate preparation. The cartoon was created with BioRender. B, densitometric analysis of HIF-1 $\alpha$ / $\beta$ -actin ratio as a hypoxia marker in male (blue bars) and female (pink bars) P5 pups from control (white bars) and L-NAME (green bars) dams. C and D, protein levels of IL-6 (C) and TNF- $\alpha$  (D) in the brain cortex as in A. Each dot represents one pup (one male and one female per litter). Data are presented as median  $\pm$  SD. \* $P < 0.05$ , \*\* $P < 0.005$  versus respective control group.

spatial memory. This preference was absent in L-NAME offspring, indicating memory impairment. In the MWMT probe trial, L-NAME offspring spent less time in the target quadrant (NW) and more time in the opposite quadrant (SE) than controls (Fig. 7F), further demonstrating deficits in spatial learning and memory.

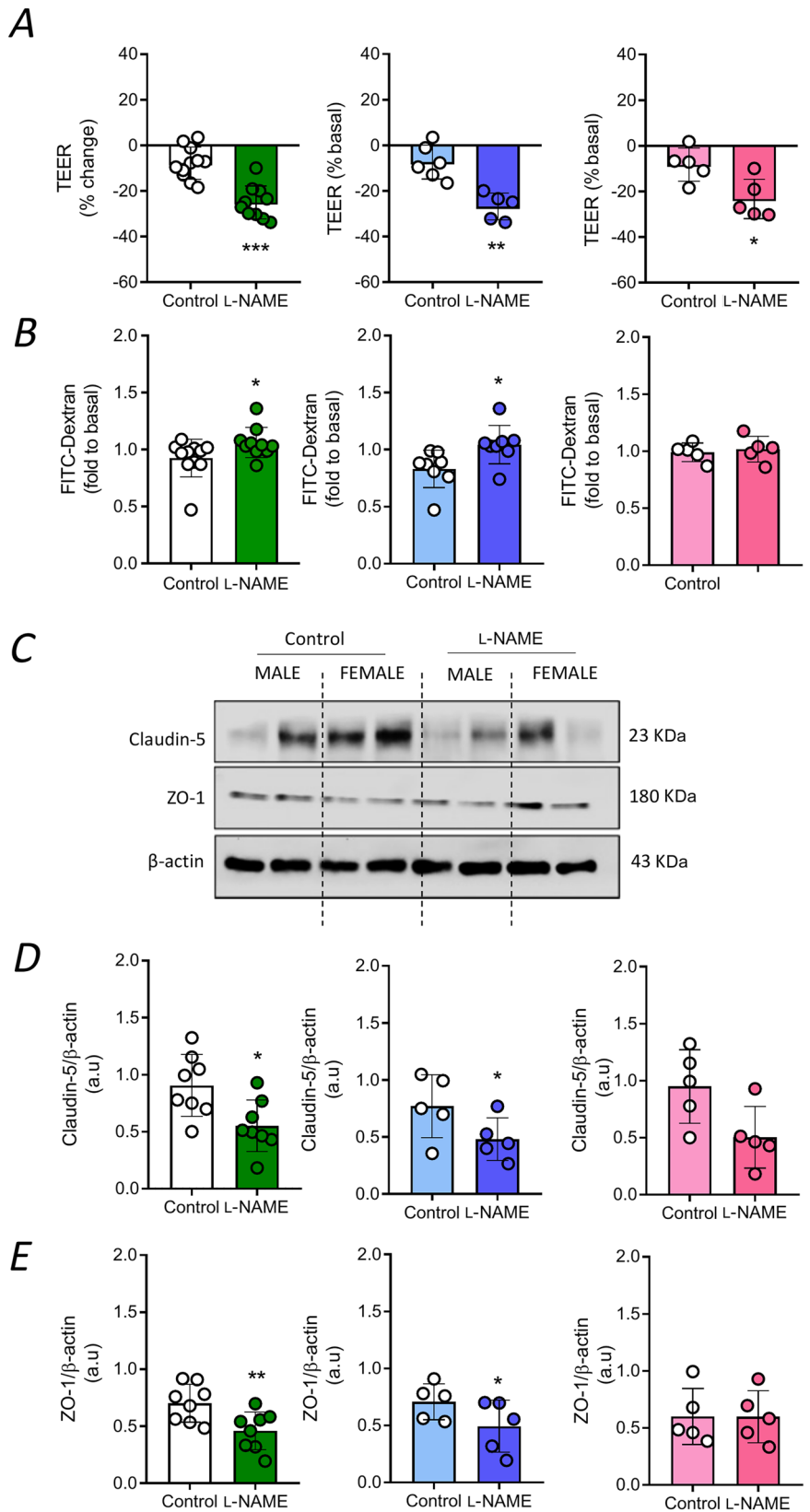
Sex-stratified analysis showed that offspring from L-NAME-treated dams had fewer crossings in the OFT than controls ( $0.9 \pm 0.9$  vs.  $3.1 \pm 1.8$  crossings per test, respectively;  $P = 0.001$ ), with similar reductions in both males and females ( $P < 0.05$  in both cases).

In the MWMT, offspring of L-NAME dams exhibited increased escape latency during the acquisition phase in both sexes (Fig. 7G). This impairment was evident throughout the 4 d analysis in females, whereas in males it became significant only during the third and fourth days (Fig. 7G). These findings indicate that prenatal exposure to L-NAME impairs both locomotor activity and spatial learning in offspring, with cognitive deficits emerging earlier and more persistently in females than in males.

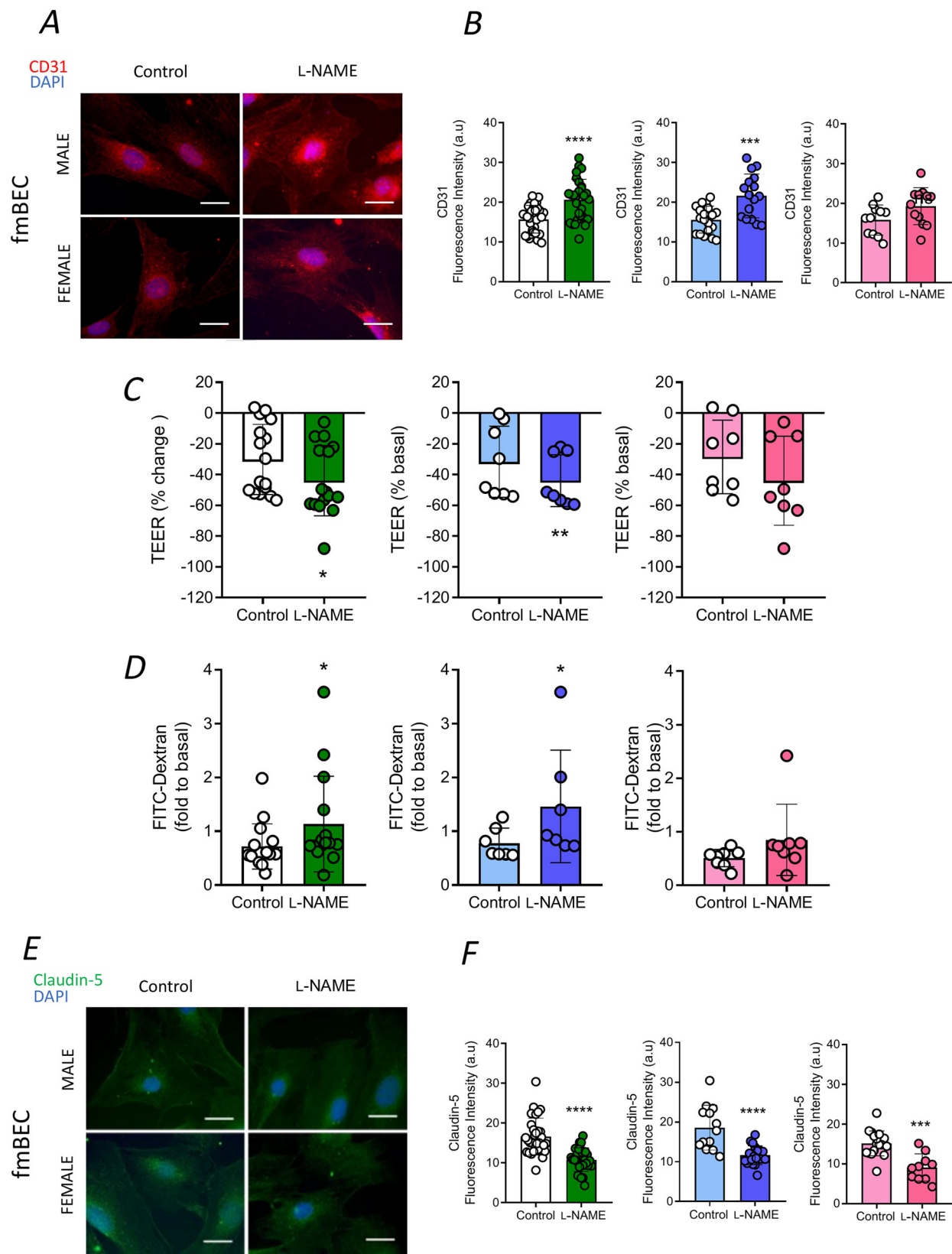


**Figure 4. Immunomodulatory serum profile in male and female offspring from preeclampsia and brain endothelial cell activation**

A, schematic showing serum collection from P5 pups of control (white bars) and L-NAME dams (green bars). The cartoon was created with BioRender. B–D, serum was analysed for circulating levels of IL-6 (B), IL-8 (C) and TNF- $\alpha$  (D) using western blot in male (blue bars) and female (pink bars) pups. E–F, serum from male (blue bars) and female (pink bars) pups was also used to stimulate mouse brain endothelial cells (Bend3) to evaluate protein levels of IL-6 (E), IL-8 (F) and TNF- $\alpha$  (G) by western blot. Each dot represents one pup (one male and one female per litter). Data are presented as median  $\pm$  SD. \* $P < 0.05$ , \*\* $P < 0.005$  versus respective control group.



**Figure 5. Serum from male offspring of preeclamptic mothers and barrier properties of Bend3**  
 Serum from P5 male (blue bars) and female (pink bars) offspring of control (white bars) and L-NAME dams (green bars) was used to stimulate Bend3 cells and assess barrier function. *A* and *B*, percentage change in transendothelial electrical resistance (TEER) (*A*) and permeability to FITC-dextran (70 kDa) in Bend3 cells (*B*) treated with serum from male and female pups of control and L-NAME dams. *C*, representative blots of tight junction proteins (Claudin-5 and ZO-1) and loading control  $\beta$ -actin in Bend3 cells treated as in *A*. *D* and *E*, densitometric analysis of Claudin-5/ $\beta$ -actin (*D*) and ZO-1/ $\beta$ -actin ratios (*E*) in Bend3 cells. Each dot represents one pup (one male and one female per litter). Data are presented as median  $\pm$  SD. \* $P < 0.05$ , \*\* $P < 0.005$  versus respective control group.



**Figure 6. Serum from offspring of preeclampsia and barrier properties of primary fetal mouse brain endothelial cells**

A, representative images of immunodetection of the endothelial activation marker CD31 in primary fetal mouse brain endothelial cells (fmBECs) exposed to serum from P5 male (blue bars) and female (pink bars) offspring

of control (white bars) and L-NAME dams (green bars). *B*, quantification of CD31 immunodetection. *C* and *D*, percentage change in transendothelial electrical resistance (TEER) (*C*) and permeability to FITC-dextran (70 kDa) in fmBECs (*D*) treated as in *A*. *E* and *F*, representative images of claudin-5 immunodetection (*E*) and quantification of claudin-5 immunofluorescence in fmBECs (*F*) treated as in *A*. Each dot represents one pup (one male and one female per litter). Data are presented as median  $\pm$  SD. \* $P < 0.05$ , \*\* $P < 0.005$ , \*\*\* $P < 0.01$ , \*\*\*\* $P < 0.0001$  versus respective control group.

## Discussion

In this study, offspring exposed prenatally to a preeclampsia-like environment induced by maternal L-NAME administration displayed both structural (reduced vascular density, fewer tip endothelial cells, decreased GLUT1) and functional (increased permeability, reduced tight-junction proteins) alterations in brain microvessels. These changes were present in both sexes, but several measures (particularly BBB permeability and endothelial activation) were more pronounced in males. Elevated cortical HIF-1 $\alpha$  and IL-6 levels further indicate a hypoxic and immunomodulatory brain milieu, consistent with previous reports of neuroinflammation in preeclampsia-like models (Giambrone et al., 2019; Katoh et al., 2022). In adulthood, both male and female L-NAME offspring exhibited cognitive impairments, with spatial learning deficits emerging earlier and more persistently in females. Together, our findings demonstrate that prenatal exposure to a preeclampsia-like environment induces lasting alterations in brain angiogenesis and BBB integrity, accompanied by long-term cognitive deficits, with distinct patterns of vulnerability between sexes.

While sex-specific effects on the developing brain following preeclampsia are increasingly recognized, data on sex differences in cerebrovascular outcomes remain limited. Preeclampsia is associated with a twofold increased risk of perinatal stroke (Li et al., 2017; Srivastava et al., 2022), with male neonates disproportionately affected. Male infants are generally more vulnerable to adverse perinatal outcomes, including severe asphyxia (Mohamed & Aly, 2014), cerebral palsy (Surveillance of Cerebral Palsy, 2000), or greater long-term IQ impairment than female infants with a similar degree of hypoxia-induced encephalopathy (Smith et al., 2014). This pattern of male-biased susceptibility is also observed in neonatal rodent models of hypoxia-ischaemia (Demarest, Schuh, Waddell, et al., 2016; Demarest, Schuh, Waite, et al., 2016; Hill & Fitch, 2012; Smith et al., 2014), as well as the fact that bacteraemia exacerbates only male exposure to perinatal hypoxia-ischaemia brain injury (Gravina et al., 2020). Animal studies further support a heightened vulnerability in males to long-term cardiovascular (Beckers et al., 2021; Lu et al., 2007) and cerebrovascular complications (Carver et al., 2014; Hofsink et al., 2023).

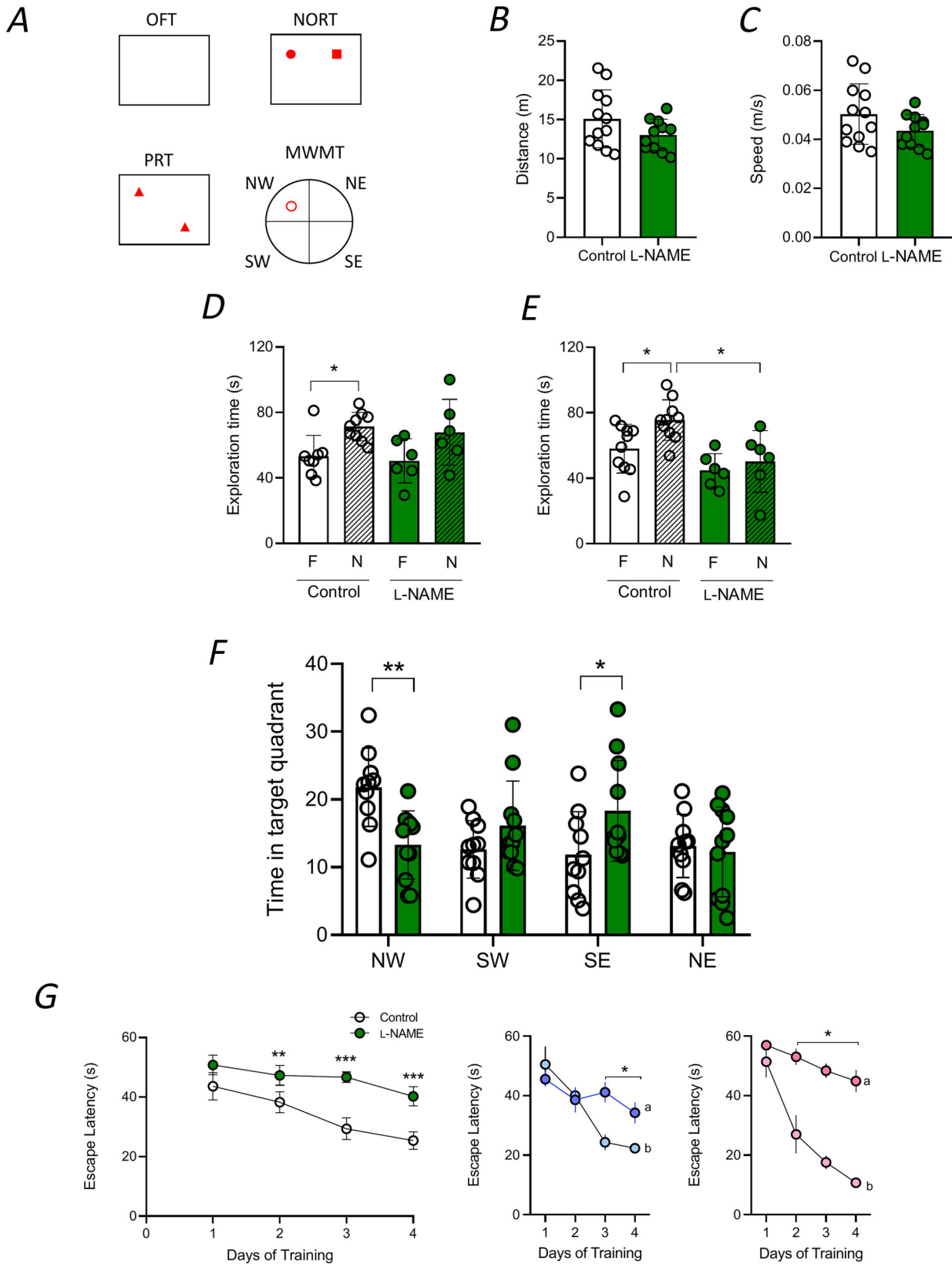
Our findings align with this literature but also highlight important nuances. Prenatally, offspring exposed to

L-NAME exhibited reduced fetal size at gestational day 19, an effect primarily seen in females. However, two-way ANOVA revealed no significant sex  $\times$  treatment interactions. Moreover, by P5, both sexes showed reduced growth, indicating a general treatment effect rather than a sex-specific one. In contrast, apparent sex-dependent differences emerged in early cerebrovascular measures. Specifically, we identified significant sex  $\times$  treatment interactions for endothelial tip-cell density and GLUT1 expression, suggesting that prenatal preeclampsia-like conditions differentially alter early angiogenic processes in males and females. For all other angiogenic, vascular and BBB-related parameters, no significant interactions were observed; treatment effects were therefore interpreted at the pooled or sex-stratified level, as appropriate.

These findings align with recent work by Wu et al. (2025), who reported long-term neurodevelopmental and behavioural impairments in L-NAME-exposed offspring, predominantly in males, associated with reduced oligodendrocyte maturation. Taken together, these studies indicate that while global developmental parameters, such as body size, may be similarly affected in males and females, the neurovascular system shows selective male-biased vulnerability. Our results support this interpretation; although both sexes exhibited impaired angiogenesis and BBB disruption, males showed more consistent deficits in tight-junction expression, endothelial activation and barrier permeability.

The mechanisms driving impaired brain vascular development in offspring exposed to preeclampsia remain incompletely understood. Our serum-transfer experiments demonstrate that circulating factors from L-NAME offspring are sufficient to impair angiogenesis and disrupt BBB integrity *in vitro*, implicating systemic mediators in postnatal cerebrovascular dysfunction. Previous work has shown that female HUVECs (human umbilical vein endothelial cells) derived from preeclamptic pregnancies exhibit broader gene dysregulation and greater TNF- $\alpha$ -induced barrier disruption than their male counterparts (Zhou et al., 2019), indicating a potential role for sex-specific endothelial responses. These data support a model in which inflammatory mediators, potentially including immunomodulatory cytokines (i.e. IL-6, TNF- $\alpha$ ), induce or exacerbate brain endothelial dysfunction in a sex-dependent manner.

Our findings indicate that serum from L-NAME offspring induces an immunomodulatory phenotype in brain endothelial cells, consistent with elevated



**Figure 7. Spatial learning and synaptic plasticity in adult offspring of preeclampsia**  
 A, cognitive performance was assessed in adult offspring from Control (white bars) and L-NAME-treated dams (green bars) using four behavioural tests: Open Field Test (OFT), Novel Object Recognition Test (NORT), Place

Recognition Test (PRT), and Morris Water Maze Test (MWMT). *B* and *C*, distance (*B*) and speed (*C*) in the OFT. *D* and *E*, NORT (*D*) and PRT (*E*) to demonstrate preference for the novel object or location (*N*) versus the familiar one (*F*). *F*, MWMT showing time in the targeted quadrants (NW, SW, SE, NE). *G*, escape latency during acquisition sessions across all offspring, and separated by male (blue bars) and female (pink bars). Each dot represents one pup (one male and one female per litter). Data are presented as median  $\pm$  SD. \* $P < 0.05$ , \*\* $P < 0.005$ , \*\*\* $P < 0.01$  versus respective control group.

circulating cytokines. Although both IL-6 and IL-8 were increased in the serum of L-NAME offspring, only IL-6 remained significantly elevated in males after sex stratification, suggesting that IL-6 may contribute to the more pronounced endothelial activation observed in males. Elevated IL-6 has been reported in several preeclampsia-like animal models (Giambrone et al., 2019; Katoh et al., 2022), whereas human data remain variable (Al-Othman et al., 2001; Reyes-Aguilar et al., 2019). Importantly, IL-6 can directly activate brain endothelial cells via JAK/STAT3 signalling (Rummel et al., 2006), providing a plausible link between systemic inflammation and BBB dysfunction. These observations support IL-6 as a potential mediator of long-term neurovascular alterations following preeclampsia-like exposure, particularly in males, although additional mediators are probably involved.

### Endothelial dysfunction and BBB impairment

We provide consistent evidence of BBB disruption in offspring from preeclampsia-like pregnancies, characterized by increased accumulation of fluorescent tracers and albumin in the brain, particularly in males. These effects were observed in both the RUPP (Lara et al., 2022) and L-NAME models and replicated *in vitro* using primary brain endothelial cells. Serum from L-NAME offspring consistently induced immunomodulatory activation of brain endothelial cells, supporting a role for circulating factors in barrier dysfunction. Moreover, reduced expression of the tight junction proteins CLDN5 and ZO-1 further implicates junctional disassembly in BBB breakdown.

This barrier leakiness, together with impaired angiogenesis, probably contributes to the hypoxic and immunomodulatory brain milieu observed in exposed offspring. In turn, hypoxia may establish a feed-forward loop, exacerbating cerebrovascular dysfunction by impairing vasodilatory signalling and angiogenic responses (Aasa et al., 2015; Guan et al., 2023), ultimately worsening BBB integrity (Mughis et al., 2024), as described in models of stroke and neurodegeneration (Mao et al., 2015). Such sustained neurovascular instability may underlie the cognitive deficits observed in adult offspring (see below), linking early BBB dysfunction to long-term neurological vulnerability. These findings highlight the need to monitor neurovascular health in children born to mothers with pre-

eclampsia and to explore early interventions to restore endothelial function and BBB integrity.

### Long-term effects on cognition in offspring from preeclampsia

Adult offspring from preeclampsia-like pregnancies exhibited hippocampal-dependent cognitive impairments, with preserved motor function. Both sexes were affected in learning and memory tasks; however, female offspring showed more pronounced deficits in the MWMT, indicating a heightened susceptibility to spatial learning deficits, which may reflect sex-specific neurovascular adaptations. These results extend previous studies reporting reduced locomotor learning performance in female offspring from pregnancies complicated with preeclampsia (Valencia-Narbona et al., 2023). Collectively, these findings support the hypothesis that prenatal exposure to preeclampsia disrupts neurodevelopmental programming of the hippocampus, with long-term consequences for memory function. Intriguingly, the more substantial cognitive impairments observed in females may reflect a differential early-life adaptation to cerebrovascular dysfunction, warranting further investigation.

We acknowledge several limitations. Although we identified early cerebrovascular alterations in offspring of preeclamptic women, we did not perform mechanistic experiments to identify the circulating factors responsible. Our cross-sectional design and the analysis of different brain regions (cortex for vascular function vs. hippocampus for cognition) further limit direct interpretation. Future longitudinal, region-specific studies are required to clarify sex-dependent mechanisms linking vascular dysfunction to cognitive outcomes in this context.

In summary, prenatal exposure to L-NAME-induced preeclampsia leads to early cerebrovascular dysfunction, characterized by BBB leakiness and impaired angiogenesis, which persists into adulthood as cognitive impairment. Male offspring display more pronounced acute vascular deficits, whereas females show some task-specific cognitive alterations, particularly in spatial learning. These findings highlight the role of early vascular programming in shaping brain development and underscore the need to identify molecular targets for early diagnosis and intervention in offspring exposed to preeclampsia.

## Perspective

The mechanisms underlying altered brain structure (Ratsep, Hickman, et al., 2016; Ratsep, Paolozza, et al., 2016) and neuronal connectivity (Figueiro-Filho et al., 2017; Mak et al., 2018) observed in children and experimental offspring exposed to preeclampsia (and their potential sex-dependent differences) are largely unknown. However, in hypoxic pups, preclinical findings indicate that male-associated disadvantages over female offspring include: less functional and structural development of the lungs and cardiorespiratory circulation (Lorente-Pozo et al., 2018); high circulating levels of testosterone associated with impaired stress response of the neonatal brain (Hill & Fitch, 2012); greater pro-apoptotic signalling pathways and neuronal cell death (Hill & Fitch, 2012); or less expression of the mitochondrial biogenesis-associated transcription factor, Nrf2/GABP $\alpha$ , leading to decreased electron transport chain proteins, and low antioxidants levels (Demarest, Schuh, Waddell, et al., 2016; Demarest, Schuh, Waite, et al., 2016). However, a critical factor, less studied in the literature, is the need for adequate CBF to maintain brain function.

Within this context, our findings demonstrate that preeclampsia induces long-lasting and potentially sex-specific alterations in the neurovascular system. Male offspring demonstrate more pronounced endothelial activation and BBB disruption, whereas female offspring exhibit task-specific cognitive alterations. These divergent outcomes may reflect intrinsic sex differences in immune programming, vascular maturation or hormonal responsiveness during early development.

Our findings suggest that circulating inflammatory and anti-angiogenic mediators may serve as biomarkers of neurodevelopmental risk in offspring exposed to preeclampsia. The persistent endothelial activation induced by offspring serum suggests that the postnatal environment remains altered, prolonged after the initial *in utero* insult. Investigating the molecular pathways underlying these vascular and cognitive impairments may yield therapeutic targets to mitigate the long-term effects of maternal preeclampsia on offspring health. We encourage future follow-up studies in children exposed to preeclampsia, utilizing cognitive analysis and structural and functional magnetic resonance imaging studies to identify vulnerabilities that require early intervention.

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## Additional information

### Data availability statement

All data supporting the findings of this study are included in the paper and its Supporting Information files. Additional datasets are available from the corresponding author, Prof. Carlos Escudero, upon reasonable request and subject to institutional and ethical approval.

### Competing interests

The authors declare that they have no conflicts of interest.

### Author contributions

C.E. conceptualized the manuscript. F.T. performed most of the experiments and analyses. J.A., H.S. and E.E.G. performed some animal and *in vitro* experiments. F.N. and E.R. performed an immunohistochemical analysis of brain angiogenesis. P.S. was a clinician, pathologist and consultant, and supported histological and immunohistochemical analysis. J.E. and O.H. performed additional brain vascular analysis by histochemistry. D.M. performed cognitive tests and electrophysiology experiments. A.A. supervised cognitive test analysis and performed electrophysiology experiments. M.V. advised on the clinical relevance of the findings and supported the experimental design. J.A. monitored the progress of experimental work. All co-authors approved the final version of the manuscript. All authors have approved the final version of the manuscript and agreed to be accountable for all aspects of the work. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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## Keywords

blood–brain barrier, brain angiogenesis, brain endothelial cells, cognitive impairments, offspring, preeclampsia

## Supporting information

Additional supporting information can be found online in the Supporting Information section at the end of the HTML view of the article. Supporting information files available:

## Peer Review History