On Line Data Supplement for

Maternal Exposure to Particulate Matter Increases Postnatal Ozone-Induced Airway Hyperreactivity in Juvenile Mice

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Methods:

Urban traffic-related particle, PM.

Urban particles (SRM 1648) were purchased from the National Institute of Standards and Technology (Gaithersburg, MD), and described in the certificate of analysis (1): "They were prepared from urban PM collected in the St. Louis, MO area in a baghouse over a period of over 12 months. The material was removed from the filter bags, combined in a single lot, screened through a fine mesh sieve to remove extraneous materials and thoroughly blended in a v-blender. The material was then packaged into sequentially numbered bottles." The major constituent elements are (mass fraction in %): Al 3.4%, Fe 3.9%, K 1.1%, and the minor constituent elements are: Na 0.43%, Pb 0.66%, Zn 0.48%. There are also metal constituents (ng/mg), including As (115), Cd (75), Cr (403), Cu (609), Mn (786), Ni (82), Se (27), U (5.5) and V (127). The particles were suspended in 0.9% NaCl for the use in these experiments.

Instillations during pregnancy

Time mated mice gestations were determined from the appearance of a copulatory plug after overnight housing of female mice with mice. Instillations were done twice weekly, two days apart, for each of the three weeks of gestation. The first instillation was on gestation days 2 or 3, and the second on day 4 or 5 and this schedule was used for all three weeks.

Pulmonary mechanics measurements

Pups were anesthetized with ketamine 50 mg/kg and xyalzine 10 mg/kg i.p., placed on a temperature controlled water blanket set at 37ºC, and connected to a continuous ECG monitor. Supplemental doses of xylazine 10 mg/kg were administered if the heart rate rose > 10% above baseline. A plastic tracheal cannula was placed into the trachea by cutdown and secured with silk sutures. This was connected to a small animal ventilator (flexiVent, SciReq, Montreal, Quebec, Canada). Airway pressure (side arm of the tracheal cannula) and tidal volume data are generated by the application of a 2-3 second sine wave volume perturbation at amplitude ~ 0.2 ml and frequency = 2.5 Hz, at PEEP = 3 cm H_2O. Following baseline resistance measurements, mice are challenged for 30 seconds with methacholine aerosol (generated by nebulization with Aeroneb Pro, SciREQ) at the indicated increasing concentrations. Between doses the lung is hyperinflated to return resistance to baseline levels prior to the next administered dose of nebulized methacholine. After baseline recruitment maneuvers to achieve total lung capacity, baseline total respiratory system resistance, R, compliance, C, and elastance, E, measurements were made using the linear single-compartment model with multiple linear regression as described by the manufacturer. Large airway (Newtonian) resistance, Rn, was estimated using the constant phase model fitted to input impedance.

Immunohistochemical detection of neutrophils. Sections were dewaxed in xylene, rehydrated in graded ethanol. After antigen retrieval using a citrate buffer according to the manufacturer's directions (Antigen Unmasking, Vector, Burlingame CA), sections were blocked in 3% goat serum in phosphate buffered-saline Tween-20® 0.05% v/v, pH 7.2 (PBS-T) for 1 hour, incubated overnight with anti-myeloperoxidase (Upstate #-07-

496) 1:200, washed, detected in goat-anti-rabbit-alkaline phosphatase 1:500(Vector), then developed using nitroblue tetrazolium/bromochloroindolyl phosphate (Roche, Indianapolis, IN) and 1 mM levamisole (Vector) according to the manufacturer's directions. Sections were counterstained with nuclear fast red (Vector).

Immunohistochemical detection of α- smooth muscle actin. Sections were quenched in 3% hydrogen peroxide in methanol for 15 minutes, then treated with antigen retrieval as noted above, blocked in 3% goat serum for one hour, overlain with rabbit monoclonal anti-α-smooth muscle actin 1:500 (Epitomics, Burlingame, CA) in 3% goat serum in PBS-T overnight at 4ºC, then detected with goat anti-rabbit biotin (1:1,000, Vector) followed by ABC*Elite* (Vector) and diaminobenzidine (Vector) according to the manufacturer's directions. Sections were counterstained with hematoxylin.

Morphometric Assessment of Alveolar Development

Two images/section were obtained at 400 × magnification with an upright microscope (E400, Nikon) using a computer generated set of random stage coordinates. Images were obtained under identical illumination with a digital camera (DP11, Olympus, Melville NY). Only images without large vessels or airways were collected. If large vessels or airways were encountered by chance, the stage was moved horizontally to acquire an image containing distal lung parenchyma. Alveolar number was estimated by converting the color images to binary images using image analysis software (Metamorph v 6.4, Molecular Dynamics, West Chester PA), then superimposing a test array of 10 × 10 dots. The number of dots overlying alveolar septal tissue was then divided by the total number

of test dots within the parenchymal space to yield alveolar volume density. Alveolar surface area was estimated by overlaying an array of test lines of known length (calibrated using a stage micrometer image) on the binary images. The number of intercepts of septal tissue with the test lines was divided by the total line length to yield alveolar surface density, as previously described in detail (2).

Figure Legends

E1. Effect of postnatal ozone ± maternal PM on lung inflammation. Images from the peripheral lung from representative sections from each treatment group that were stained with hematoxylin and eosin. High-power inset shows predominance of mononuclear cells.

E2. Effect of postnatal ozone ± maternal PM on neutrophil influx: myeloperoxidase immunolabeling (purple) with nuclear fast red counterstain.

E3. Effect of postnatal ozone ± maternal PM on airway smooth muscle bulk. Representative photomicrographs from each treatment group demonstrate brown diaminobenzidine which indicates α-smooth muscle actin immunolabeling.

E4. Effect of postnatal ozone ± maternal PM on mucous metaplasia. Representative color photomicrographs from each treatment group show periodic acid-Schiff, Alcian blue stained airway epithelial cells, corresponding to Figure 6 in the print version (*arrows*).

References

E1. Reed WP. Standard reference material 1648, urban particulate matter. 1991. Available from: http://ts.nist.gov/MeasurementServices/ReferenceMaterials/archived_certificates/1648.%20Aug%2030,%201991.pdf.

E2. Auten RL, Mason SN, Whorton MH, Lampe WR, Foster WM, Goldberg RN, Li B, Stamler JS, Auten KM. Inhaled ethyl nitrite prevents hyperoxia-impaired postnatal alveolar development in newborn rats. *Am J Respir Crit Care Med* 2007;176:291-299.

Figure E1

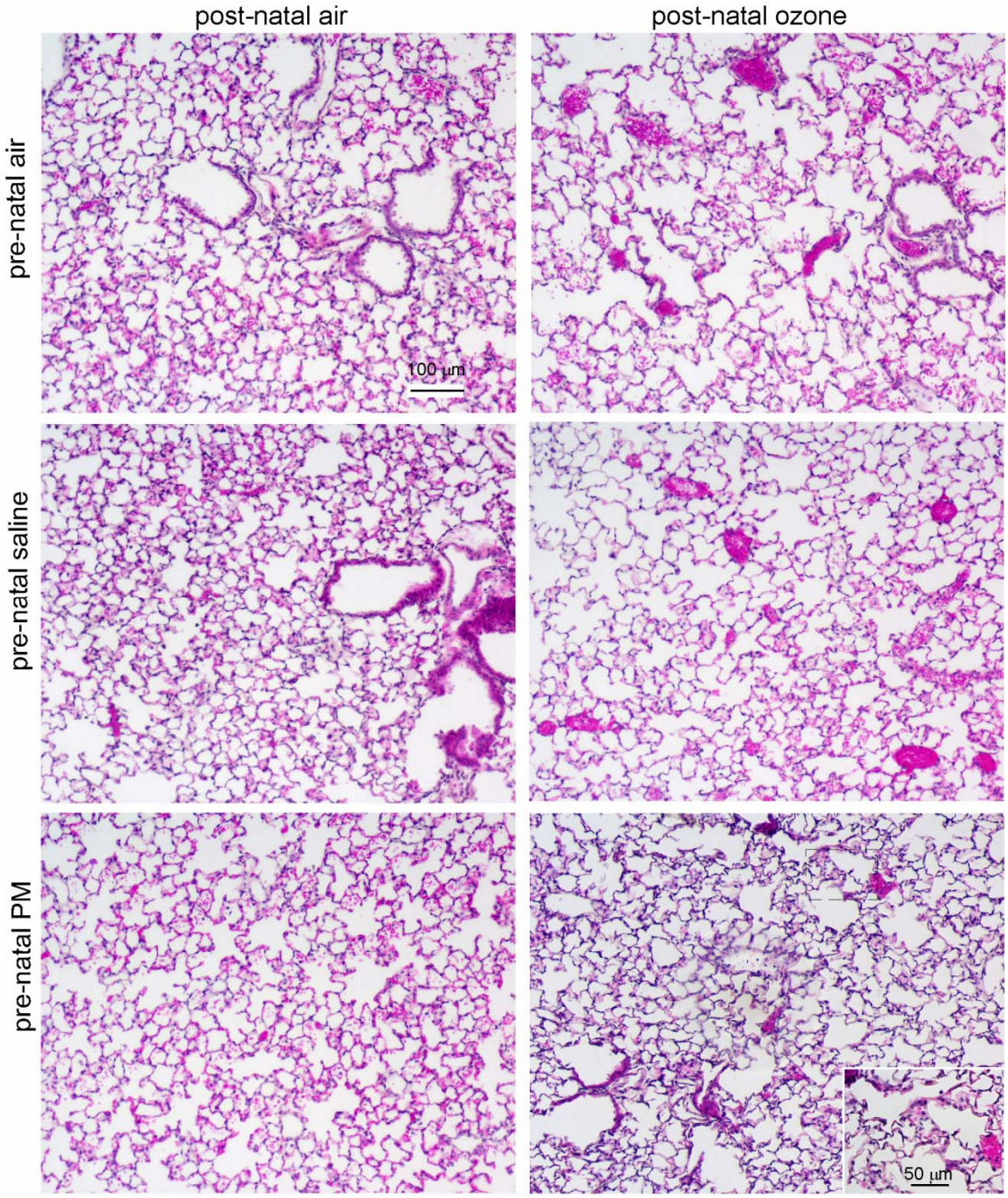

post-natal air
post-natal ozone
pre-natal air
pre-natal saline
pre-natal PM
100 µm
50 µm

Figure E2

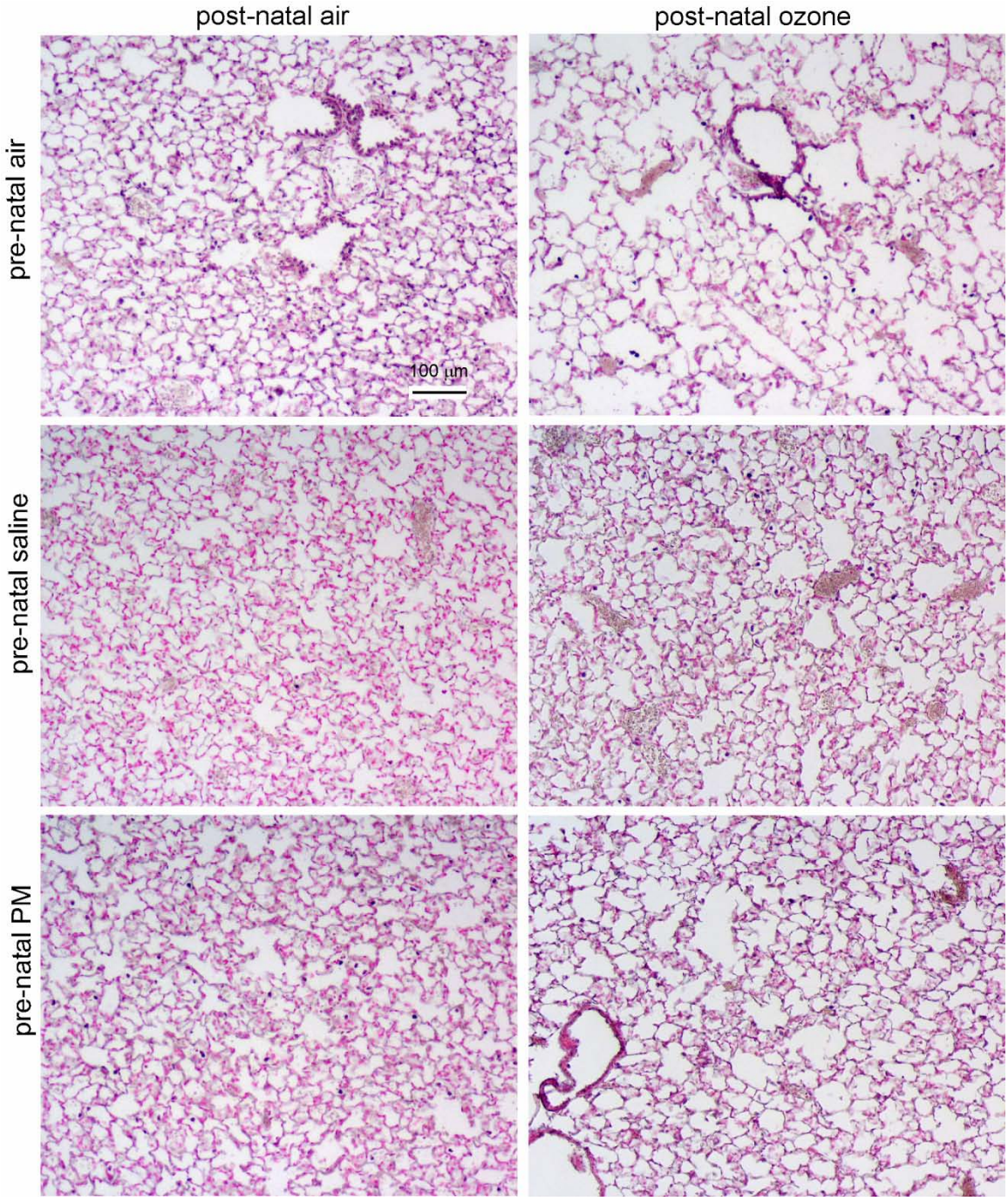

post-natal air
post-natal ozone
pre-natal air
pre-natal saline
pre-natal PM
100 μm

Figure E3

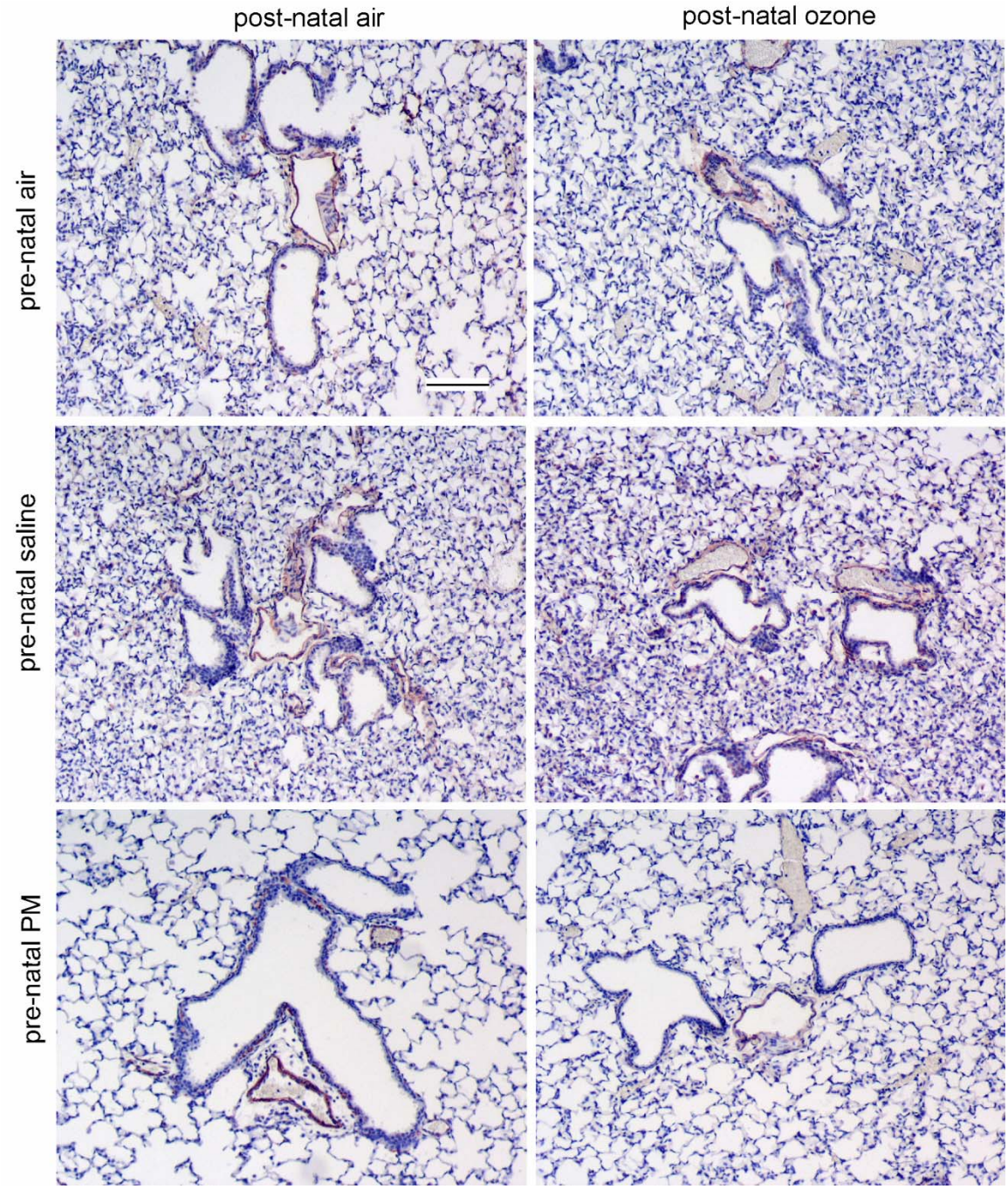
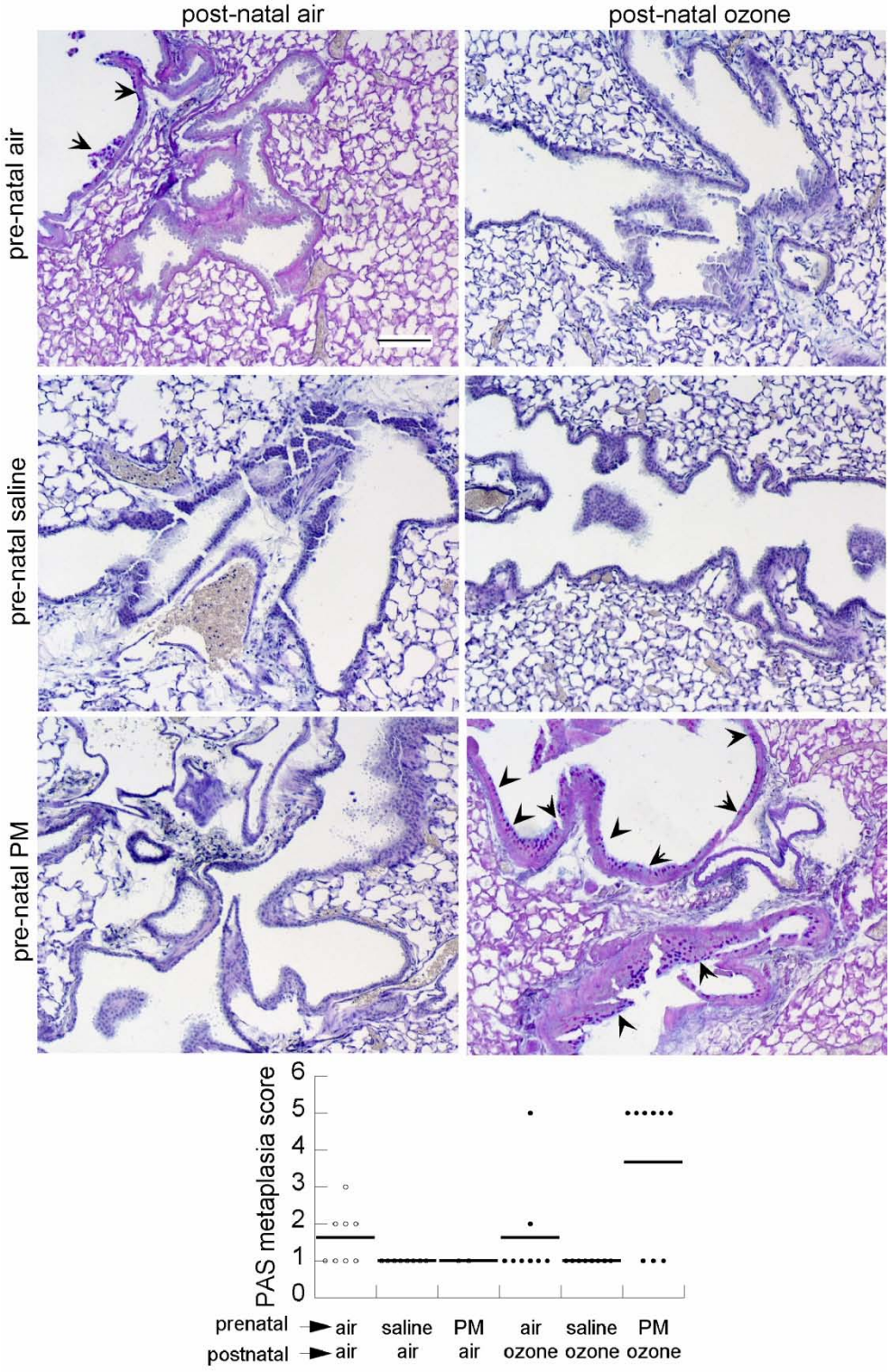

post-natal air
post-natal ozone
pre-natal air
pre-natal saline
pre-natal PM

Figure E4


post-natal air
post-natal ozone
pre-natal air
pre-natal saline
pre-natal PM
PAS metaplasia score
0
1
2
3
4
5
6
prenatal
postnatal
air
air
saline
air
PM
air
air
ozone
saline
ozone
PM
ozone