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## Abstract

Millions of children are exposed to concentrations of air pollutants, including fine particulate matter (PM<sub>2.5</sub>), above safety standards. Mexico City Metropolitan Area (MCMA) megacity children show an early brain imbalance in oxidative stress, inflammation, innate and adaptive immune response-associated genes and blood-brain-barrier breakdown. We investigated serum and cerebrospinal fluid (CSF) antibodies to neural and tight junction proteins and environmental pollutants in 139 children ages 11.91±4.2 y with high v low air pollution exposures. We also measured metals in serum and CSF. MCMA children showed significantly higher serum actin IgG, occludin/zonulin 1 IgA, IgG, myelin oligodendrocyte glycoprotein IgG and IgM (p<0.01), myelin basic protein IgA and IgG, S-100 IgG and IgM, and cerebellar IgG (p<0.001). Serum IgG antibodies to formaldehyde, benzene, and bisphenol A, and concentrations of Ni and Cd were significantly higher in exposed children (p<0.001). CSF MBP antibodies and nickel concentrations were higher in MCMA children (p=0.03). Air pollution exposure damages epithelial and endothelial barriers and is a robust trigger of tight junction and neural antibodies. Cryptic 'self' tight junction antigens can trigger an autoimmune response potentially contributing to the neuroinflammatory and Alzheimer and Parkinson's pathology hallmarks present in megacity children. The major factor determining the impact of neural antibodies is the integrity of the blood-brain barrier. Defining the air pollution linkage of the brain/ immune system interactions and damage to physical and immunological barriers with short and long term neural detrimental effects to children's brains ought to be of pressing importance for public health.

**Keywords:** Alzheimer, air pollution, children, innate and adaptive immunity, neurodegeneration, neuroinflammation, particulate matter, tight junction and neural reactive autoantibodies.

## 1. Introduction

Air pollution is a serious health problem in megacities around the world [1, 2]. Mexico City Metropolitan Area (MCMA) children with no known risk factors for neurological or cognitive disorders exhibit significant deficits in a combination of fluid and crystallized cognition tasks versus children in low pollution cities [3-5]. Brain structural and volumetric changes are seen in both MCMA children and young animal facility dogs [3, 4]. The dogs' frontal white matter lesions are characterized by vascular sub-cortical pathology associated with neuroinflammation, gliosis, and ultrafine particulate matter (UFPM) deposition [3]. Similarly, our previous pediatric studies showed that prefrontal white matter hyperintensities are associated with neuroinflammation as well as with pronounced cognitive deficits [4-6]. MCMA children more likely showed impaired attention, short-term memory and learning abilities that are commonly seen in neurodegenerative disorders [4]. In association to these cognitive symptoms, young MCMA residents exhibit the neuropathological hallmarks of Alzheimer and Parkinson's diseases i.e., amyloid beta 42 ( $A\beta_{42}$ ) plaques, tau hyperphosphorylation with pre-tangles and  $\alpha$ -synuclein accumulation [6-9]. Thus, concerning current challenges for mechanisms of neuroinflammation and neurodegeneration, we suspected the association between neurocognition, immune system and barrier damage may be relevant for the extensive and critical debate on the contribution of air pollutants to neurodegeneration [5, 6, 9].

We have overwhelming data in the literature supporting the detrimental human systemic and neuroinflammatory responses to urban air pollution and similar results have been recorded in experimental animals exposed to different air pollution components [10-19]. A key component of the air pollution effects in both human and animals is the breakdown of the nasal, olfactory, blood-brain-barrier (BBB) and alveolar-capillary barriers and the expression of detrimental genes [20-25]. Exposures to tobacco smog containing a combination of particulate matter (PM) and gases is associated with membrane structural changes to tight junction (TJ's) protein complexes and cilia and increased permeability of the lung/blood barrier [26, 27]. The GI tract barrier is also compromised and recent research links inflammatory bowel diseases, changes in gut microbiome, and abdominal pain with air pollution [28-33]. Breakdown of epithelial and endothelial barriers, including the BBB are common in the air pollution scenario. Thus, it is plausible that brain inflammation and epithelial/endothelial barrier breakdown are accompanied of immune responses characterized by antibodies against membrane-bound, intracellular or secreted proteins [34-42]. The role of powerful pro-inflammatory cytokines i.e., Interleukin- 1  $\beta$  is crucial to induce the production and accumulation of complement factors (like C1q) that facilitate neutrophil entry and BBB breakdown and amplification of immune cell recruitment as in the case of neuromyelitis optica [41].

With this background, we focused on the issue of TJ's and neural autoimmunity in megacity pediatric populations, a critical subject given that more megacities are rising globally. We know that in central nervous system (CNS) autoimmune disorders the BBB breakdown has been considered as key step in the disease process, and since MCMA children exhibit epithelial and endothelial barrier breakdown, including supra and infratentorial BBB breakdown, our working hypothesis establishes that exposed children will have a profile of brain autoantibodies targeting key neural components. Given that MCMA children have lifetime exposures to high concentrations of fine particulate matter ( $PM_{2.5}$ ) associated with metals related to combustion, the issue of metals impacting directly the CNS is also very important [43].

In this proof-of-principle study, we hypothesized that in highly exposed children the alteration of their adaptive immune system associated to extensive breakdown of epithelial and endothelial barriers would result in formation of antibodies to tight junction, neuronal and glial proteins. The primary aim of this study was to measure serum tight junction and neural antibodies and combustion metals from MCMA children v controls from low pollution cities (i.e., criteria pollutants below the current US standards). Concurrently, given that cerebrospinal fluid (CSF) is a window of the brain milieu, we also explored neural antibodies and metals in CSF control v exposed samples. Finally, in keeping with the fact urban children are highly exposed to a wide variety of environmental pollutants, including endotoxins and endocrine disruptors we also measured antibodies to well-known environmental toxicants.

Our results identify serum and CSF autoantibodies against neuronal and glial-derived proteins. Tight junction antibodies are plausible evidence of the significant epithelial and endothelial damage associated with exposure to air pollutants and its components, while the acquired immune system response to environmental pollutants highlights its role as an early responder. Our results suggest that an impaired tolerance to key neural and extra-neural antigens in urban children in association with a disrupted BBB could translate in a neurovascular unit (NVU) failing to assure an optimal crosstalk between the periphery and the brain [44, 45]. In this scenario, a metabolically dysregulated microenvironment associated with NVU/BBB dysfunction could contribute to the significant changes in oxidative stress, inflammation, innate and adaptive immune responses and the early AD histopathological hallmarks described in MCMA children [6, 9]. Future studies will be aimed to determine the serum and CSF antibodies' diagnostic value in exposed children and if serum antibodies could represent accessible biomarkers of neurotoxic effects readily applicable to pediatric populations at risk of neural air pollution effects, including neurodegenerative changes.

## 2. Materials and Methods

### 2.1. Study Cities and Air Quality

Children's cohorts were selected from the Mexico City Metropolitan Area (MCMA) and small cities in Mexico (Zacatlán and Huachinango, Puebla; Zitácuaro, Michoacán; Puerto Escondido, Oaxaca; Chalma, Veracruz; Tlaxcala, Tlaxcala). The control cities have <100,000 inhabitants and are characterized by concentrations of the six criteria air pollutants (ozone, particulate matter, sulfur dioxide, nitrogen oxides, carbon monoxide and lead) below the current US EPA standards [46]. Our largest control source was Polotitlán, in the Mexico State, 121 km north-northwest of Mexico City and at 7500 ft above sea level. Polotitlán has 13,000 inhabitants, mostly dedicated to agricultural and bovine milk production. There is a very restricted industrial production, including one concrete and one candle small factories. The geological characteristics include volcanic rock formation with basaltic and andesitic rocks high in Fe, Mg and Ca. A major highway connecting Mexico City with Queretaro runs 5 miles away from the town center.

Mexico City Metropolitan Area is an example of extreme urban growth and accompanying environmental pollution [47-51]. The metropolitan area of over 2,000 km<sup>2</sup> lies in an elevated basin 7400 feet above sea level surrounded on three sides by mountain ridges. MCMA nearly 20 million inhabitants, over 40,000 industries, and >4 million vehicles consume more than 40 million liters of petroleum fuels per day, producing an estimated annual emission of 2.6 tons of particulate and gaseous air pollutants [52]. MCMA motor vehicles produce abundant amounts of primary fine PM<sub>2.5</sub>, elemental carbon, particle-bound polycyclic aromatic hydrocarbons, carbon monoxide, and a wide range of air toxins, including lipopolysaccharides, formaldehyde, acetaldehyde, benzene, toluene, and xylenes [53-55]. The high altitude and tropical climate facilitate ozone production all year and contribute to the formation of fine secondary particulate matter. Air quality is worse in the winter, when rain is scanty and thermal inversions are frequent. Children from MCMA were residents in the northern-industrialized and southern-residential zones. Southern Mexico City children have been exposed to significant concentrations of ozone, secondary tracers (NO<sub>3</sub><sup>-</sup>) and PM-LPS, while northern children have been exposed to higher concentrations of volatile organic compounds (VOCs), PM<sub>2.5</sub>, and its constituents: organic and elemental carbon including polycyclic aromatic hydrocarbons, secondary inorganic aerosols (SO<sub>4</sub><sup>2-</sup>, NO<sub>3</sub><sup>-</sup>, NH<sub>4</sub><sup>+</sup>), and metals (Zn, Cu, Pb, Ti, Mn, Sn, V, Ba) [48,50,55]. Recent studies on the composition of PM<sub>2.5</sub> with regards to sites and samples collected in 1997 show that composition has not changed during the last decade [48].

### 2.2 Participants

This research was approved by the research ethics committee of the Hospital Central Militar in Mexico City. Children gave active assent and their parents gave written informed consent to participation in the study. This work includes data from 139 children 74F, 65M (*Mean age* =11.91yrs, *SD* =4.2). There were two groups of children included in this study: Group 1 (n: 28, *Mean age* =10.46 y, *SD* =4.2, low pollution 8F/6M; high pollution 6F/8M) corresponded to children admitted to the hospital from either MCMA or a low polluted city with a work up diagnosis of lymphoblastic leukemia entering a clinical protocol, which included a spinal tap. Group 1 includes hospitalized children with normal CSF samples. None of the selected Group 1 children had previous oncologic and/or hematologic treatments. Group 2 (n: 111, 44 controls (24F/20M), 67 MCMA (33F/34M), *Mean age* =13.37 y, *SD* =4.2) were clinically healthy children from MCMA and control cities and their serum samples were taken as part of their annual pediatric examination during a longitudinal follow-up. These healthy children did not have a spinal

tap and thus no CSF samples were available. Inclusion criteria for all participating children were: negative smoking history and environmental tobacco exposure, lifelong residency in MCMA or a control city, residency within 5 miles of the city monitoring stations, full term birth, and unremarkable clinical histories prior to either hospital admission (for Group 1 children) or to recruitment into the clinically healthy longitudinal study. MCMA children in this study use mostly public transportation, including the subway system. Low and high pollution exposed children were matched by age, gender and socioeconomic status.

### 2.3 CSF Samples

Spinal tap was performed in the supine position from lumbar levels using a standard 22 spinal needle. CSF was collected dripping in free air in 1 ml aliquot into Nalge Nunc polypropylene CryoTubes. Lumbar puncture samples were collected during non-traumatic, non-complicated procedures. CSF pleocytosis was defined as CSF white blood cell (WBC) counts of  $>7$  cells per  $\text{mm}^3$ .

### 2.4 Peripheral Blood Samples

Blood was collected from an antecubital vein using a 21-G needle. After centrifugation at 3,000 rpm for 10 min, aliquots of 1.5 ml serum were transferred to CryoTubes and samples were frozen at  $-20$  °C and then transferred to  $-80$  °C and stored until further analysis.

### 2.5 Inductively Coupled Plasma Mass Spectrometry ICP-MS Metal Analyses

The ICP-MS analysis was performed with the Agilent ICP-MS 7700cx and the CETAC AS-500 sampler using a peristaltic pump. Certified metal standards used were from Agilent Multielement Calibration standards, and RECIPE reference standards for blood and plasma. Because no control reference material was available for CSF, we used plasma reference materials for the quality control of the CSF samples (Supplemental Table 1). Calibration with 0.6-M  $\text{HNO}_3$  solutions of matrix-matched multimetal standards was performed. To check for instrumental drift, multimetal standards with known metal concentrations were analyzed for every ten samples. Certified reference materials were analyzed at the beginning and end of each analytical sequence. Resolution mode for each isotope was selected for each run in order to obtain maximum sensitivity with minimum RSD (relative standard deviation of measurement). Metals analyzed were (isotopes used in the quantification given in parentheses) aluminum (Al 27), arsenic (As 75), calcium (Ca 44), cadmium (Cd 111), chromium (Cr 52), copper (Cu 63), cobalt (Co 59), iron (Fe 56), lead (Pb 208), mercury (Hg 200), magnesium (Mg 25), manganese (Mn 55), molybdenum (Mo 98), nickel (Ni 60), selenium (Se 82), vanadium (V 51), and zinc (Zn 66).

### 2.4 ELISA reagents

Astrocytic protein S100-B was purchased from EMD Biosciences, San Diego, CA 92121 US. Myelin basic protein (MBP), actin, elastin, lipopolysaccharide (LPS), mercury, mixed heavy metals, tolylene-2,4-diisocyanate, formaldehyde, dinitrophenol, and bisphenol-A, were purchased from Sigma Aldrich, St. Louis, MO 63103, US. HPLC grade peptides for MOG, occludin/zonulin, cerebellar tissue, lung epithelial cell, glucose regulated protein-3, and matrix metalloproteinase-3 were synthesized by Bio-Synthesis, Lewisville, TX 75057 US, with a purity of greater than 80%.

### 2.5 Preparation of Benzene Ring HSA Conjugate.

For this preparation, 40 mg of P-aminobenzoic acid was dissolved in 2 mL of 1 N HCl and cooled by immersion in an ice bath. In parallel, one gram of HSA was dissolved in boric acid 0.16 M sodium chloride (0.15 M buffer pH 9.0 [pH was raised with NaOH]). The beaker containing the solution of albumins was surrounded by an ice bath on a magnetic stirrer. The solution of diazonium salt was added dropwise, with rapid stirring, to the cold protein solution. After addition of each drop, the pH was readjusted to 9.0 to 9.5 with NaOH. After adding all the solution, the reaction was allowed to continue with slow stirring for at least an hour with further additions of NaOH solution, and maintenance of the pH at the range of 9.0 to 9.5. Unreacted small molecules were removed by extensive dialysis or by passage through a column of Sephadex G-25 in the cold room with an isotonic salt solution as an eluting buffer [56].

## 2.6 Preparation of Formaldehyde-Human Serum Albumin (F-HSA).

Briefly, 1 mg of BS or HSA (Biocell Carson, CA) in PBS, pH 7.4, each separately, were exposed to 1 mg of formaldehyde (Fisher Scientific, Fairlawn, NJ). The mixture was incubated for 30 min at 37°C and was then extensively dialyzed against PBS. The F-HSA was sterilized with a 0.2 µm filter (Millipor Corp., Bedford, MA). Electrophoretic and immunoelectrophoretic comparison of HSA with F-HSA was performed to determine conjugation occurrence. Conjugation was evidenced by altered mobility of F-HSA, when compared with HSA.

## 2.7 Preparation of Toluene-2,4-Diisocyanate-Human Serum Albumin (TDI-HSA).

One gram of HSA was dissolved in 10 mL of a buffer solution containing potassium chloride (0.05 mol/l), sodium borate (0.05 mol/l), pH 9.4, and cooled to 4°C. Dioxane (10 mL) containing 0.15 mL of toluene-2,4-diisocyanate was then added dropwise while stirring over a period of 3 h, followed by addition of 2 mL of ethanolamine, centrifugation, dialysis filtration, and lysophilization.

## 2.8 Binding of Mercury, Mixed Heavy Metals to HSA.

For this preparation, 100 mg of human serum albumin (HSA) was dissolved in 9 mL of buffer solution containing potassium chloride and sodium borate 0.05 mL/liter and pH was adjusted to 9.4 with 0.1 N NaOH. Then 25 mg of Thimerosal, mercury chloride or other heavy metal was dissolved in 1 mL of buffer and added dropwise to the HSA solution. The reaction mixture was stirred overnight, dialyzed against 0.1 M PBS using tubing with a cutoff of 8,000 Dalton. Conjugation of haptenic chemicals was confirmed by sodium dodecyl sulfate (SDS) gel electrophoresis and shift in HSA band. In addition, spectrographic analysis of the conjugate was undertaken. In all cases there was a marked increase in absorption from 230 to 260 nm, which indicated that haptenic chemicals became covalently linked to the HSA or protein carrier.

## 2.9 Detection of Antibodies by ELISA

Antigens and peptides were dissolved in PBS or methanol at a concentration of 1.0mg/ml, then diluted 1:100 in 0.1 M carbonate-bicarbonate buffer, pH 9.5, and 100 µL were added to each well of a polystyrene flat bottom ELISA plate. Plates were incubated overnight at 4°C and then washed three times with 200 µL Tris-buffered saline (TBS) containing 0.05% Tween 20, pH 7.4. The non-specific binding of immunoglobulins was prevented by adding a mixture of 1.5% bovine serum albumin (BSA) and 1.5% gelatin in PBS, and incubated overnight at 4°C. Plates were washed as described above, and then 100 µL of serum samples from controls and patients diluted at an optimal dilution of 1:100 in 0.1M PBS Tween containing 2% BSA were added to duplicate wells and incubated for 1h at room temperature. Sera from patients with celiac disease and neuroimmune disorders with known high titers of antibodies were used as positive controls. Plates were washed, and then alkaline phosphatase goat anti-human IgG, IgM or IgA F(ab')<sub>2</sub> fragments (KPI, Gaithersburg, MD) optimal dilution of 1:400-1:2000 in 1% HSA-TBS was added to each well; plates were incubated for an additional 1h at room temperature. After washing five times with TBS-Tween buffer, the enzyme reaction was started by adding 100µL of paranitrophenylphosphate (PNPP) in 0.1 ml diethanolamine buffer 1mg/ml containing 1mM MgCl<sub>2</sub> and sodium azide pH 9.8. The reaction was stopped 45 min later with 50 µL of 1N NaOH. The optical density (OD) was read at 405 nm. To detect non-specific binding, several control wells contained all reagents except human serum. For each tested antigen, serial dilution was conducted on 12 sera containing different levels of antibodies (4 low, 4 medium, 4 high). A decline in optical densities proportional to the respective dilutions was observed.

## 2.10 Coefficients of Intra- and Inter-Assay Variation

Coefficients of intra-assay variation were calculated by running 5 samples eight times within a single assay. Coefficients of inter-assay variation were determined by measuring the same samples in six consecutive assays. This replicate testing established the validity of the ELISA assays, determined the appropriate dilution with minimal background, and detected serum antibodies against different antigens. Coefficients of intra- and inter-assay variations for antibodies against all tested antigens and peptides were less than 12%.

### 2.11 Data Analysis

We first calculated the sample mean and sample standard deviation of each of the characteristic variables including the measurements of the antibodies and the metals, in control and the Mexico City groups. Next, we calculated the p-values of the two-sample t-tests to investigate whether the sample means of the variables are significantly different between the groups. Please note, in that statistical test, we assumed that the measurements of each variable in each group roughly follow Gaussian distribution. To correct for multiple comparisons we performed the Simes-Bonferroni correction  $\alpha(m+1)/2m$ , where  $m$ =number of comparisons [57]. As a result of such correction, we concluded that the sample means of a variable in the two groups are significantly different only if the corresponding p-value is smaller than the adjusted threshold value of 0.027. Next, we calculated Pearson's correlation coefficients (PCC) among each pair of the variables in each group and in the pooled data irrespective of groups. PCC measures how well the relationship between two variables can be described by a linear function. We also calculated approximate p-values for the significance of PCCs. We concluded that a PCC is significantly different from zero only if the corresponding p-value is smaller than 0.05. Finally, we performed a two way cluster analysis of the PCCs of the serum antibodies with environmental antibodies and metal concentrations in both Controls and Mexico City children. We carried out the above mentioned statistical analyses in the statistical software 'R' (<http://www.r-project.org/>).

## 3. Results

### 3.1 Air Quality

Substantial reductions in the concentrations of some criteria pollutants (such as lead, CO and SO<sub>2</sub>) have been achieved in MCMA through the development and implementation of comprehensive air quality management and evaluation programs [48]. Despite these important gains, MCMA residents remain exposed year-round to concentrations of airborne pollutants exceeding ambient air quality standards, especially PM and ozone. Particulate matter is broadly defined by the diameter of the aerodynamic particles, and classified into coarse particles (<10 to >2.5 $\mu$ m; PM<sub>10</sub>), fine particles (<2.5 $\mu$ m, PM<sub>2.5</sub>) and ultrafine PM (UFPM<100nm). Fine and ultrafine PM are of particular interest given their capability to reach the brain [8, 16,17]. Routine measurements of PM<sub>2.5</sub> in MCMA began in 2004, however, there is evidence that high levels of PM<sub>2.5</sub> were common before this year [47]. While the 24-hour PM<sub>2.5</sub> NAAQS of 35  $\mu$ g/m<sup>3</sup> is still exceeded in the northern area of MCMA, the respective annual primary NAAQS of 12  $\mu$ g/m<sup>3</sup> has been historically exceeded across the metropolitan area [52]. Figures 1 and 2 show the trend of observed 24-hour PM<sub>2.5</sub> concentrations at two representative sites of MCMA relevant to this study. The Pedregal site is a residential urban area located in the southwest of MCMA and the Xalostoc site an industrialized and populated area in the north east of this megalopolis. In addition, a review of the historical trend of the four highest daily maximum ozone eight-hour average concentrations for each of three consecutive years in southwest Mexico City shows that the levels until 2012 have kept close or above the O<sub>3</sub> NAAQS standard in the last 23 years depending on the urban zone. Figure 3 shows the trend of ozone 8-hour average concentrations for the Southwest and Northeast areas of MCMA from 1989 through 2012. The current 8-hr average ozone NAAQS is 75 ppb. MCMA children have been exposed chronically to significant concentrations of fine PM during their entire life, including the prenatal period. The peak of PM<sub>2.5</sub> concentrations coincide with the times children are outdoors during the school recess and physical education periods and the highest ozone levels when they play outdoors at home [58,59]. All other criteria pollutants for MCMA, including carbon monoxide, nitrogen dioxide, sulfur dioxide and lead were below the current EPA standards (data not shown). Control children have been lifelong residents in low pollution cities with all criteria air pollutants below the US EPA NAAQS standards.

### 3.2 Serum Data

The results of the 17 selected antibodies are shown in Table 1. Our antibody panel was divided in three categories: tight junction, neural and environmental antibodies. The sera from 111 children (*Mean age* =13.37 y, *SD* =4.2), 67 children from MCMA and 44 controls, were measured for the simultaneous presence of IgA, IgG and IgM antibodies against the selected antigens. There was a significant statistical difference between MCMA v control children for 13/ 17 antibodies, the concentrations being higher in Mexico City children. High affinity IgG and IgA actin and occludin-zonulin antibodies showed significant differences between exposed and control children. Myelin oligodendrocyte glycoprotein, myelin basic protein, S-100, and cerebellar antibodies were significantly higher in exposed children. IgA antibodies against glucose-regulated protein 78, an endoplasmic reticulum (ER) homologue

of HSB70 which plays a dual role in the ER controlling protein folding in order to prevent aggregation and regulating the signaling of the unfolded protein response [60] were higher in MCMA children. Antibodies against common environmental pollutants i.e., formaldehyde, benzene, bisphenol A, and metals were also higher in MCMA children. For four antigens including elastin, lung epithelial antigen, matrix metalloproteinase 3 and lipopolysaccharide (LPS), the results showed no significant differences between cohorts.

In addition to a significant elevation in antibodies against heavy metals, when metal serum concentrations were measured, there was a significant statistical difference between MCMA children v controls for Ag, Ca, Cu, Fe, Hg, Ni, Se and Zn, with higher concentrations in MCMA children (Table 2). While controls had statistically higher values for Al, Cd, Mn, Mo, and V, the higher concentrations of these metals is likely associated with naturally occurring metals in the earth's crust and related to the agricultural soil characteristics, the individual distribution coefficient K<sub>d</sub> values and their solid liquid distribution. [61, 62] A potential Cd and V source for control children under certain meteorological conditions could be related to emissions from the Tula refinery located 71.2 km east from Polotitlán [63-64].

### 3.3 CSF Data

CSF samples were clear, colorless, with a normal opening pressure, a mean WBC count of  $2.1 \pm 1$  cells per  $\text{mm}^3$  and no RBC. Glucose was  $55.9 \pm 6.7$  mg/100 ml in controls and  $54.8 \pm 11.7$  mg/100 ml in Mexico City children ( $p=0.3$ ). All CSF samples were classified as normal. Table 3 shows the results of the selected antibodies in CSF samples of Mexico City v controls. MBP IgG and IgA concentrations were significantly higher in Mexico City children ( $p=0.03$ ), while S-100 and MBP IgM showed no statistical significance. The results of the selected metal CSF concentrations are seen in Table 4. Nickel was significantly higher in CSF samples of Mexico City children v controls ( $p=0.03$ ), while As, Cr, Cu, Hg, Mn and V were not significantly different. Supplemental Tables 2A and 2B, 3A and 3B show the significant Pearson correlations and  $p$ -value  $< 0.05$ , between the neural and TJ's antibodies, environmental antibodies and metal concentrations in Controls (Supplemental Tables 2A and 2B) and MCMA (Supplemental Tables 3A and 3B) children respectively. Mexico City children had significant Pearson's correlations between neural antibodies and all cell junction proteins explored. Actin antibodies showed significant correlation with S-100, MOG and MBP antibodies and also strongly correlated with cerebellar antibodies. Of interest was the observation that a few metals correlated with neural antibodies, including Cr, Co, Pb (Supplemental Table 3 B). Antibodies to common environmental pollutants such as tolylene-2,4-diisocyanate, benzene, metals and LPS were strongly correlated to neural antibodies (Supplemental Table 3 B), while age exhibited no correlations with any variable. Control children also showed significant correlations between tight junction proteins and common environmental pollutants and neural antibodies (Suppl Tables 2A, 2B). Controls showed significantly higher concentrations of Al, Cd, Mn, Mo, and V in serum (Table 2) along with robust correlations with neural antibodies, notably V and MBP IgG (.47), Cd and cerebellar IgG (.46) and Cr and cerebellar IgG (.40) (Supplemental Table 2A). Figure 4 shows the cluster analysis of the serum antibody results in controls and exposed children. Tables 5 and 6 show the significant Pearson's correlations coefficients and  $p$  values respectively between the CSF neural antibodies and metals in control and MCMA children. Mexico City children showed a significant correlation between Cu CSF concentrations, S100 IgA/IgG ( $p=0.003$ ) and MBP IgA/IgG ( $p=0.004$ ), while V concentrations correlated with S100 IgA/IgG ( $p=0.03$ ). Control children showed no significant correlations between CSF metals and neural antibodies.

## 4. Discussion

Urban children with lifetime exposures to environmental air pollutants, including fine particulate matter ( $\text{PM}_{2.5}$ ) above current safety standards are showing significant increases in serum high affinity antibodies to tight junction and key neural proteins, along with antibodies to common environmental pollutants. A pivotal finding in highly exposed urban children is the CSF presence of antibodies to myelin basic protein. Also of critical importance are the results of metal combustion and subway commuter markers such as nickel showing significantly higher concentrations in MCMA serum and CSF samples [65].

These findings center the core of our discussion on five critical issues: 1. The air pollution-associated damage of epithelial and endothelial cell junction components resulting in the breakdown of critical barriers and the exposure of antigens to the immune system machinery in young children 2. The significance of antibodies to tight junctions (TJ's) and neural proteins in the presence of a disrupted BBB 3. The development of immune tolerance dysfunction and autoimmunity in children and their direct neural effects 4. The impact of systemic and CSF metals in the setting



of neuroinflammation and a disrupted BBB and their neurodegenerative consequences, and 5. The growing recognition of the role of systemic and neural inflammation and the interplay between immunity, neurodegeneration and maladaptive activation of innate/adaptive immunity as key pathogenic phenomena in Alzheimer disease.

It is well known that epithelial surfaces in direct contact with polluted air including the nasal and olfactory epithelia are extensively compromised with significant breakdown of their barrier protective functions [10, 20, 21, 66-69]. Damage to epithelial TJ's result in a breakdown of the paracellular barrier and alters selective transport pathways [70]. We also know that particulate matter activates endothelial cells, generates oxidative stress [71] and disrupts endothelial cell barrier via calpain-mediated TJ's protein degradation [72]. Of key importance for our work, is the report in a BBB model showing diesel exhaust particles (DEP) impair endothelial progenitor cells and severely compromise the integrity of endothelial cells during oxygen-glucose deprivation [73]. Brain microvascular endothelium express TJ's complexes that restrict paracellular passage across the BBB, thus any alteration to TJ's complexes will result in impaired brain functions[74].The breakdown of epithelial and endothelial barriers not only alters the paracellular flux and permeability between adjacent cells and allows for the entrance of PM and a myriad of toxins and xenobiotics, but also allows for the exposure of the TJ's multiprotein complex and extracellular matrix components to the immune system and the production of autoantibodies. The significant elevations of high affinity autoantibodies against barrier forming proteins in urban children are critical to our understanding of air pollutant mechanistic pathways affecting epithelial and endothelial barriers and the subsequent compromise of the BBB and the neurovascular unit [75-81]. Previous studies suggest that PM accesses the brain and the brainstem by the uptake of ultrafine particles through the olfactory neurons and cranial nerves such as the trigeminal (nasal) and vagus (heart and gastrointestinal tract) [3, 6-10, 20, 23, 31, 33]. These findings are a critical global issue as more children are being exposed to air pollution due to the increasing number of megacities around the world. We have previously shown in Mexico City children the extensive supra and infra-tentorial breakdown of the TJ's integrity in brain endothelial capillaries, accumulation of nanosized PM in endothelial cells (EC) and the transfer of PM from intraluminal macrophages to EC in brain capillaries [82, 6]. Ultrafine and fine PM resulting from combustion products rich in organic chemicals and inorganic components, including heavy metals and endotoxin have a high pro-oxidative potential and inflammatory capacity[79]. Nanosized PM can cross barriers by an endocytic pathway, can penetrate through cell membranes and through tissue resulting in severe cellular inflammatory reactions and toxicity to epithelial and endothelial barriers [79]. Outstandingly, hypoxia is also known to damage the BBB [74], this is a potentially parallel problem to MCMA children: high concentrations of the potent vasoconstrictor endothelin-1 is a robust marker of urban exposure in our pediatric cohorts [11].

Thus, in the complex scenario of sustained lifelong exposures to high concentrations of fine and ultrafine PM [50, 83-85], the presence of antibodies against barrier forming proteins notably occludin/zonulin and actin cytoskeleton could represent an additional pathway for the disruption of critical barriers [86,87]. The issue is a serious concern for the BBB because the effects impact two functionally distinct compartments: a. the so called physiological BBB, formed by capillaries ranging in diameter 4–8  $\mu\text{m}$  and consisting of a single layer of endothelia, gliovascular membrane, and the astrocytes end feet; and b. the neuroimmunological BBB, formed by postcapillary venules 10–60  $\mu\text{m}$  in diameter and two layers - the endothelium and its basement membrane and the glia limitans with associated astrocyte end feet - separated by the perivascular space [88].The impact for the former will be for small solutes while the transport of macromolecules and the diapedesis of immune cells will be associated with the neuroimmunological BBB [88].

So, what is the significance of TJ's and neural proteins autoantibodies in the presence of a disrupted BBB? It will depend essentially of the major target site: capillaries or postcapillary venules or both [88]. The anatomical target is key to link with the process of leukocyte entry into the CNS parenchyma, a process taking place at the postcapillary venules [88] and very importantly, the activation and movement of myeloid cell populations operating in the CNS [89]. Brain damage common denominator is the recruitment of circulating immune cells with the consequent innate immune response orchestrated by resident microglia, monocytes, macrophages and dendritic cells [89]. The association of neural antibodies and pathogenicity with a leaky BBB could be very important for highly exposed children [3, 82] and as Levin and coworkers suggested a "*defective BBB allows access of autoantibodies to targets on the brain cells*" [34]. If indeed the major factor determining the impact of the brain antibodies is the integrity of the BBB [34, 35, 90-93], then their presence in urban children highlights the importance of innate and adaptive immune responses, neuroinflammation and systemic inflammation as active and relentless contributors to the neuronal, glial and endothelial damage and the allowance of nuclear/cytoplasmic and membranous brain antigens to the immune system in childhood [16, 94, 95]. Of major impact is the fact that antibodies to critical myelin and glial

proteins are significant in exposed children, indicating the urgent need to fully grasp the meaning of these antibodies in a developing, inflamed brain, showing volumetric and structural changes along with emerging misfolded neural proteins [3, 4, 7, 8, 9, 82, 96]. MOG, MBP and S-100 antibodies are present in classic autoimmune disorders such as multiple sclerosis and CNS lupus [97-103], and although their quantification plays an important role in clinical practice in a number of disorders [37, 93, 104-107], only few patients with demyelinating diseases can be characterized based on their antibody profile [102, 108]. Furthermore, a growing body of evidence implicates the immune dysregulation in neurodevelopmental disorders like autism [36, 37, 42, 93, 105, 146-153]. The immune dysregulation in children is critical as the presence of brain-targeted antibodies have shown to be associated with more impaired behavioral, cognitive and adaptive traits than in children without them [148].

This concern obligates us to discuss the development of immune tolerance and autoimmunity in air pollution exposed children and their neural consequences. Myelin oligodendrocyte glycoprotein, myelin basic protein, and S-100 autoantibodies along with CSF myelin basic protein antibodies are all significantly higher in exposed children. Most of the proteins in CNS myelin are not found in peripheral myelin and myelin proteins are considered tissue-specific antigens (TSAs) [98], therefore the significance of the distinct antibodies in our cohorts is key in the context of the children's age and environmental exposures. Specifically, MOG is very small component of the myelin complex that fails to elicit any central tolerance in transgenic mice [109, 110], while MBP is an abundant CNS and peripheral myelin component and elicits both central and peripheral tolerance [98]. Escaping central tolerance without triggering T cell activation leaves the T cells vulnerable to activation by viral infections [98], making the issue of the participation of multiple layers of tolerance and different biologically plausible pathways [111, 112] to prevent CNS immunity a critical subject in urban children. The emerging idea of the development of immune tolerance breakdown and autoimmunity in our exposed cohorts will obligate us to explore a direct correlation with brain effects, including behavioral deficits [100, 113]. The interplay between genetic susceptibility and high exposures to potential triggers at an early age could gradually result in the loss of a balanced auto-tolerance and the development of CNS lesions associated with cognitive and structural alterations. In addition, recent studies show that the expression of immune-related genes and transcriptional profiles of immune cells such as T cells are governed by the epigenetic modifications that begin its establishment in utero and are heavily influenced by the environment such as the presence of the microbiota [163, 164]. Activation of the innate immunity and the subsequent instruction to the adaptive immune system to respond to the antigen recognition could result in chronic pro-inflammatory conditions, a scenario that takes place in the brain's aging process [114]. Specifically, immune/inflammation related changes develop during the course of cognitively normal aging and involve brain anatomical specific areas [114] but the mechanism behind this relationship is still unclear. This is critical for our work, since we have shown that the prefrontal cortex in highly exposed children shows a significant up-regulation of network clusters including IL1 $\beta$ , NF $\kappa$ B, TNF, IFN and TLRs [6]. Strikingly, the process of dysregulation of the innate and adaptive immune responses in MCMA children starts very early and goes hand in hand with cognitive deficits, structural and volumetric brain changes and the misfolded proteins associated with Alzheimer and Parkinson's diseases [3-5, 8-13, 82].

In this complex scenario, metal homeostasis is critical and the impact of systemic and CSF metals in the setting of neuroinflammation and a disrupted BBB adds to the systemic immune dysregulation and the CNS innate imbalance [3, 4, 9, 10, 82]. Immunomodulation of innate and adaptive immunity by metals, including metallic nanoparticles has been described in the literature [115-121]. The finding of increased concentrations of combustion-associated metals i.e., nickel, raises questions about the induced inflammatory responses playing a pivotal role in the CNS autoimmunity and inflammation [122]. Specifically, Ni has been associated to activation of a novel innate immune signaling pathway: NLRP3-ASC-caspase-1-IL-1 $\beta$  [122]. Apoptosis, ROS generation and oxidative stress with activation of caspases and bax/bcl are part of the cytotoxicity produced by Ni and other metals [123, 124, 125]. Of special concern for our children is the report on the role of Toll receptors in the Zn/Ni inflammatory responses in endothelial cells [126]. The metal-related inflammatory vascular damage is of utmost importance in view of the simultaneous presence of autoantibodies to cell junction proteins, BBB breakdown and the activation of inflammasomes in their frontal cortex [6]. Moreover, there is a clear demonstration that inhalation exposure to traffic pollution degrades TJ proteins and alters BBB permeability with associated neuroinflammation [127].

In our study, control children had several metals with higher concentrations in serum v MCMA children, an interesting observation given that our control cohort and comparable control children exhibit no neurological, cognitive or brain MRI abnormal findings [4, 5, 7]. Moreover, when brain metals have been measured in autopsy samples from comparable age, gender, high v low air pollution exposures match cohorts, manganese, nickel and

chromium are the only metals that differentiate high v low exposures in frontal samples [43]. This is key, controls may have high soil exposures to some heavy metals but their BBB is intact [3], metals [43] are not exhibiting high brain and CSF concentrations and equally important low pollution-exposed children show no cognitive and neurological deficits or brain structural and volumetric changes [4,5,7]. However, in spite of the lack of neurological and cognitive morbidity in control children, we are observing robust serum V, Cd and Cr correlations with MBP and cerebellar IgG antibodies. Thus, since V has been associated with hypomyelination [128], and produces behavioral alterations and CNS myelin deficits in neonatal rats [129], Cd ions interact with the A $\beta$ 1-42 peptide and have neural toxic effects [130,131], and Cr produces significant brain oxidative damage [132], we cannot dismiss our metal correlations in controls and the possibility that if these children move to highly polluted areas CNS harmful effects will take place once their BBB is compromised.

The CSF poses special considerations in terms of metals, as nicely reviewed by Michalke and Nischwitz [133]. CSF metal concentrations are significantly lower than serum due to the tight blood-CSF control passage [133, 134]. Metal binding forms (species) and their interaction with CNS cellular components [133] are critical to the understanding of CNS damage mechanistic pathways in a developing brain. MCMA children showed significant positive correlations between CSF Cu and CSF S100 and MBP autoantibody concentrations, while V in CSF correlated with S100 antibodies. Control children showed no significant correlations between CSF metals and brain antibodies. We agree with Michalke's group that CSF elemental speciation analysis is a critical issue in understanding the role of metals in neurodegenerative diseases, an issue of utmost importance in our pediatric cohorts. Higher CSF concentrations of Ni is of interest in our teen MCMA cohorts because in addition to its presence in PM and their exposure using the subway system, popular cell phones are potential sources of Ni [65,135,136].

There is a growing recognition of the role of systemic and neural inflammation and the interplay between immunity, neurodegeneration and maladaptive activation of innate/adaptive immunity as key pathogenic phenomenon in AD [114, 137-141]. Similar to neurodevelopmental disorders like autism, neurodegenerative diseases such as Alzheimer are also strongly associated to immunological dysregulation [141-143] and brain reactive antibodies shown to enhance intraneuronal deposition of amyloid beta 42 in the context of a damaged BBB may be a risk factor for the initiation or progression of AD [144]. The presence of brain autoantibodies in urban children with well documented systemic inflammation, increased cerebral expression of potent pro-inflammatory cytokines and chemokines and activated inflammasomes, along with the early hallmarks of AD and PD obligates us to carefully look at the interaction between the different neural compartments, their responses to air pollution and the evolving concept that indeed neurodegenerative pathology could represent an active host response or an environmental adaptation [3-14, 145]. In this context, the "*double-edged sword*" [145] of a fine balance between protective and detrimental effects as a response to air pollution in a developing brain becomes a disquiet issue.

Of particular concern for urban children and teens are the reports of the association of circulating neural antibodies with cognitive and behavioral changes, eating disorders, autism spectrum disorders, psychosis, epilepsy, and schizophrenia [36, 37, 42, 93,105, 146-153]. Also of concern are the significant down regulation of the prion cellular protein [154], the high concentrations of macrophage inhibitory factor (MIF) and the activation of TLR and inflammasomes in Mexico City children [6, 13] in compounding the autoimmune scenario [155-158]. Finally, antibodies against glucose-regulated protein 78 could contribute to decrease badly needed neuroprotection since GRP78 plays a role in apoptosis and cell survival [159].

## 5. Looking Forward

Autoimmune disease affects a significant percentage of the world population [35, 56]. Despite controversy regarding the mechanistic pathways involved in the CNS damage associated with neural antibodies [34, 35], one thing is very clear: the integrity of the neurovascular unit is critical [35, 88, 142, 160], thus we fully agree with the Levin and Diamond groups that in the presence of BBB compromise, neural antibodies might contribute to initiation and/or pathogenesis of a wide spectrum of neurological diseases. The issue is important in the developing brain because the damage may have short and long term consequences. The key question regarding our findings remains whether the presence of neural autoantibodies in the serum and CSF of highly exposed children could play a role in the mechanistic pathways conducive to neuroinflammation and neurodegeneration on one hand and to cognitive deficits and structural and volumetric brain changes observed in these children. Screening for neural antibodies ought to be included in future longitudinal studies to determine their profile, prevalence and progression as the child

grows in the polluted environment. Moreover, neural antibodies are to be tested on specific brain regions using appropriate tissues [151]. There is a need for looking into the early autoimmune responses to air pollution and the evolving clinical, cognitive and structural/volumetric brain changes. Immune dysregulation associated to air pollutant exposures could become a global epidemic threat as megacities are rising and thus millions of children become targets of immune-mediated neural pathologies.

## **6. Summary**

Fine tuning of immune-to-brain communication is crucial to neural networks appropriate functioning, thus evidence of barrier disruption associated with neural autoimmunity is a subject of deep concern for urban children. A large body of work on the immune system, brain developmental programming, autoimmunity, brain-reactive antibodies and disease already exists [34, 35, 94-100, 106-108, 114, 137, 142-144, 161] expanding this knowledge in the scenario of air pollution pediatric effects could greatly facilitate our understanding of the downstream mechanisms of the complex interaction of antigens and targets and to elucidate if the humoral responses are the culprit of pediatric neurological and cognitive morbidity.

Future studies will determine the diagnostic and prognostic value of the presence of neural antibodies in urban children and if they could represent accessible biomarkers of neurotoxicity readily applicable to urban populations at risk of CNS pollution effects. Neural immunity might contribute to the neuroinflammatory and neurodegenerative changes present in highly exposed children. Defining the linkage and the health consequences of the brain/ immune system interactions in the developing brain chronically exposed to air pollutants ought to be of pressing importance for public health.

## **7. Declaration of Conflicting Interests**

The authors declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

## **8. Acknowledgement of author's contributions**

L. Calderón-Garcidueñas planned and directed the study, wrote the manuscript, edited and formatted the manuscript. A. Vojdani performed the antibody measurements and contributed to the editing of the paper. Eleonore Blaurock-Busch, Yvette Busch, and Albrecht Friedle did the metal analysis and contributed to the editing of the paper. Maricela Franco-Lira obtained patient consent and ethical approvals, got the samples and contributed to the editing of the paper. Partha Sarathi Mukherjee performed the statistical analysis and wrote the manuscript. Su-Bin Park contributed to write and edit portions of the paper. Ricardo Torres-Jardón contributed with his air pollution expertise, prepared the pertinent figures and wrote the paper. Amedeo D'Angiulli contributed to the statistical analysis, wrote, edited and formatted the manuscript. All authors approved the final draft of the manuscript for publication. All authors had complete access to the study data that support the publication.

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**Table 1.** Antibodies results in serum samples in Control versus Mexico City Metropolitan Area (MCMA) children (Optical Density Units ODU, Mean  $\pm$ SD)

Variables	Control Mean	SD	Mexico City Mean	SD	t value	p value
S100 IgA	0.354	0.283	0.355	0.187	-0.030	0.976
S100 IgG	0.455	0.197	0.911	0.678	<b>-4.469</b>	<b>0.0003</b>
S100 IgM	0.540	0.230	0.813	0.478	<b>-3.560</b>	<b>0.0006</b>
MOG IgA	0.536	0.168	0.589	0.286	-0.946	0.347
MOG IgG	0.5023	0.167	0.771	0.307	<b>-5.321</b>	<b>0.000001</b>
MOG IgM	0.698	0.274	0.971	0.622	<b>-2.777</b>	<b>0.007</b>
MBP IgA	0.515	0.119	0.715	0.336	<b>-3.871</b>	<b>0.0002</b>
MBP IgG	0.519	0.247	1.013	0.679	<b>-4.732</b>	<b>0.00001</b>
MBP IgM	0.669	0.167	0.766	0.315	-1.872	0.065
CEREB IgA	0.511	0.177	0.618	0.347	-1.891	0.063
CEREB IgG	0.546	0.232	0.846	0.448	<b>-4.117</b>	<b>0.0001</b>
CEREB IgM	0.684	0.293	0.915	0.571	<b>-2.483</b>	<b>0.015</b>
OZ IgA	0.457	0.098	0.603	0.310	<b>-3.097</b>	<b>0.003</b>
OZ IgG	0.482	0.152	0.582	0.168	<b>-3.043</b>	<b>0.003</b>
OZ IgM	0.652	0.311	0.856	0.639	-1.990	0.051
Actin IgA	0.596	0.198	0.680	0.262	-1.754	0.083
Actin IgG	0.822	0.325	1.019	0.362	<b>-2.799</b>	<b>0.006</b>
Actin IgM	0.724	0.225	0.873	0.357	<b>-2.439</b>	<b>0.017</b>
Elastin IgA	0.215	0.064	0.270	0.191	-1.873	0.067
Elastin IgG	0.347	0.312	0.354	0.273	-0.117	0.907
Elastin IgM	0.627	0.487	0.689	0.431	-0.658	0.512
LungEC IgA	0.863	0.230	0.956	0.269	-1.803	0.075
LungEC IgG	1.072	0.243	1.118	0.248	-0.915	0.362
LungEC IgM	1.160	0.213	1.185	0.315	-0.449	0.655
MMP3 IgA	0.262	0.06	0.345	0.189		0.17
MMP3 IgG	0.444	0.157	0.528	0.282		0.06
MMP3 IgM	0.517	0.2	0.638	0.405		0.60
GRP IgA	0.247	0.083	0.322	0.184	<b>-2.578</b>	<b>0.012</b>
GRP IgG	0.349	0.166	0.376	0.195	-0.733	0.465
GRP IgM	0.531	0.531	0.542	0.331	-0.131	0.896
TDI IgA	0.208	0.035	0.231	0.115	-1.354	0.181
TDI IgG	0.335	0.090	0.379	0.079	<b>-2.541</b>	<b>0.013</b>
TDI IgM	0.267	0.073	0.301	0.145	-1.439	0.155
Formald IgA	0.256	0.065	0.373	0.271		0.58
Formald IgG	0.434	0.162	0.794	0.488	<b>-4.840</b>	<b>0.00001</b>
Formald IgM	0.488	0.314	0.634	0.370		0.07
BenzenelgG	0.561	0.235	0.739	0.354	<b>-2.905</b>	<b>0.005</b>
Benzene IgM	0.476	0.210	0.550	0.326		0.52

BPA IgA	0.293	0.08	0.377	0.2		0.12
BPA IgG	0.466	0.181	0.836	0.504		<b>0.0001</b>
BPA IgM	0.395	0.148	0.509	0.343		0.25
LPS IgA	0.448	0.244	0.497	0.242	-0.983	0.328
LPS IgG	0.574	0.201	0.626	0.304	-0.992	0.324
LPS IgM	0.618	0.281	0.719	0.430	-1.359	0.178
MHM IgA	0.243	0.056	0.293	0.156	<b>-2.046</b>	<b>0.045</b>
MHM IgG	0.422	0.161	0.658	0.390	<b>-3.864</b>	<b>0.0003</b>
MHM IgM	0.354	0.193	0.462	0.266	<b>-2.282</b>	<b>0.025</b>
Hg Ig	0.228	0.050	0.281	0.133	<b>-2.568</b>	<b>0.013</b>
Hg IgG	0.531	0.235	0.866	0.568	<b>-3.782</b>	<b>0.0004</b>
Hg IgM	0.352	0.116	0.495	0.331	<b>-2.819</b>	<b>0.007</b>

Astrocytic protein S100, Myelin oligodendrocyte glycoprotein MOG, Myelin basic protein MBP, Cerebellar antigen, occludin/zonulin OZ, Actin, Elastin, Lung epithelial antigen, Matrix Metalloproteinase 3, MMP3, Glucose-regulated protein 78, GRP78, tolylene-2.4-diisocyanate TDI, Formaldehyde, Benzene, Bisphenol A, BPA, lipopolysaccharide LPS, Mixed Heavy Metals MHM, Mercury Hg.

**Table 2.** Metal concentrations in serum samples, controls v Mexico City Metropolitan Area. Data are expressed in µg/L

Variables	Control Mean	SD	Mexico City Mean	SD	t value	p value
Ag	0.124	0.145	0.412	0.647	<b>-2.618</b>	<b>0.012</b>
Al	90.051	36.254	33.006	23.057	<b>7.478</b>	<b>1.53E-09</b>
As	0.592	0.288	0.606	0.424	-0.161	0.872
Ca	66.366	21.020	98.177	20.557	<b>-6.221</b>	<b>4.81E-08</b>
Cd	0.050	0.028	0.024	0.029	<b>3.703</b>	<b>0.0004</b>
Co	0.383	0.081	0.415	0.116	-1.308	0.1956
Cr	1.865	0.840	1.571	1.457	1.031	0.3068
Cu	0.910	0.211	1.199	0.277	<b>-4.842</b>	<b>8.33E-06</b>
Fe	0.984	0.334	1.294	0.562	<b>-2.795</b>	<b>0.0069</b>
Hg	0.121	0.182	0.372	0.381	<b>-3.539</b>	<b>0.0008</b>
Mg	16.319	2.251	20.292	2.716	<b>-6.546</b>	<b>1.10E-08</b>
Mn	182.283	103.523	3.923	1.309	<b>9.436</b>	<b>2.42E-10</b>
Mo	1.501	0.391	1.202	0.268	<b>3.551</b>	<b>0.0008</b>
Ni	4.087	1.365	5.868	1.390	<b>-5.264</b>	<b>1.83E-06</b>
Pb	2.195	1.004	2.370	3.289	-0.307	0.7603
Se	65.486	10.702	105.620	22.968	<b>-9.439</b>	<b>5.93E-13</b>
V	1.136	0.592	0.434	0.277	<b>5.975</b>	<b>5.49E-07</b>
Zn	0.766	0.099	1.381	0.480	<b>-7.578</b>	<b>3.07E-09</b>

**Table 3.** Antibodies in CSF samples in Control versus Mexico City Metropolitan Area MCMA children (Optical Density Units ODU, Mean  $\pm$ SD)

	CONTROLS	MCMA	p value
S-100 IgG+IgA	0.438 $\pm$ 0.078	0.681 $\pm$ 0.658	0.050
S-100 IgM	0.201 $\pm$ 0.039	0.278 $\pm$ 0.247	0.098
MBP IgG+IgA	0.347 $\pm$ 0.092	0.550 $\pm$ 0.486	0.030
MBP IgM	0.392 $\pm$ 0.100	0.392 $\pm$ 0.121	0.991

**Table 4.** Metal concentrations in cerebro-spinal-fluid CSF Controls v Mexico City Metropolitan Area MCMA. Data are expressed in  $\mu$ g/L (Mean  $\pm$ SD)

Metal	Control	MCMA	p value
Arsenic	1.726 $\pm$ 1.590	2.419 $\pm$ 4.231	0.424
Chromium	1.408 $\pm$ 2.591	2.558 $\pm$ 4.046	0.214
Copper	13.323 $\pm$ 9.143	20.248 $\pm$ 19.010	0.091
Mercury	0.220 $\pm$ 0.319	0.116 $\pm$ 0.208	0.163
Manganese	1.358 $\pm$ 2.123	1.418 $\pm$ 2.486	0.923
Nickel	1.217 $\pm$ 2.579	4.193 $\pm$ 6.488	0.031
Vanadium	0.372 $\pm$ 0.565	0.308 $\pm$ 0.425	0.637

**Table 5.** Pearson correlations coefficients between CSF neural antibodies and metals, Control v Mexico City Metropolitan Area MCMA children

CSF Control	Pearson's correlation coefficients						
	As	Cr	Cu	Hg	Mn	Ni	V
S100IgG.IgA	-0.00728	-0.29312	0.18868	0.359662	-0.2023	0.252477	-0.23699
S.100IgM	-0.1158	-0.07584	-0.03343	0.044559	-0.29735	-0.3528	-0.22511
MBPIgG.IgA	-0.27013	-0.18111	-0.22062	-0.08403	-0.02196	0.017013	0.037787
MBPIgM	0.113425	0.041454	0.052809	0.22889	-0.13442	-0.03351	-0.00175
<b>Mexico City</b>							
S100IgG.IgA	-0.06052	0.083574	<b>0.540962</b>	0.040144	0.169121	-0.21729	<b>0.394452</b>
S.100IgM	-0.18027	-0.05357	0.295591	-0.11395	0.183481	-0.2227	0.0571
MBPIgG.IgA	-0.03385	-0.06983	<b>0.521525</b>	0.074835	0.223339	-0.25808	0.278675
MBPIgM	0.051935	0.069931	0.157966	-0.11264	0.069556	-0.14873	0.074924

**Table 6.** Pearson correlations p values between the CSF neural antibodies and metals, Control v Mexico City Metropolitan Area children

CSF Control	p-values of the Pearson's correlation coefficients						
	As	Cr	Cu	Hg	Mn	Ni	V
S100IgG.IgA	0.971254	0.137851	0.345927	0.065382	0.311569	0.203894	0.233953
S.100IgM	0.565158	0.706942	0.868543	0.825333	0.131991	0.071074	0.258924
MBPIgG.IgA	0.172974	0.365972	0.268794	0.676879	0.913407	0.932876	0.851563
MBPIgM	0.573226	0.83734	0.793627	0.250805	0.503836	0.868214	0.993081
<b>Mexico City</b>							
S100IgG.IgA	0.759677	0.672434	<b>0.002955</b>	0.839277	0.389611	0.266704	<b>0.037787</b>
S.100IgM	0.358628	0.786583	0.12672	0.563698	0.349998	0.254682	0.772884
MBPIgG.IgA	0.864232	0.724026	<b>0.004426</b>	0.705084	0.253279	0.184856	0.151001
MBPIgM	0.792967	0.723636	0.422075	0.568216	0.725064	0.450059	0.704748



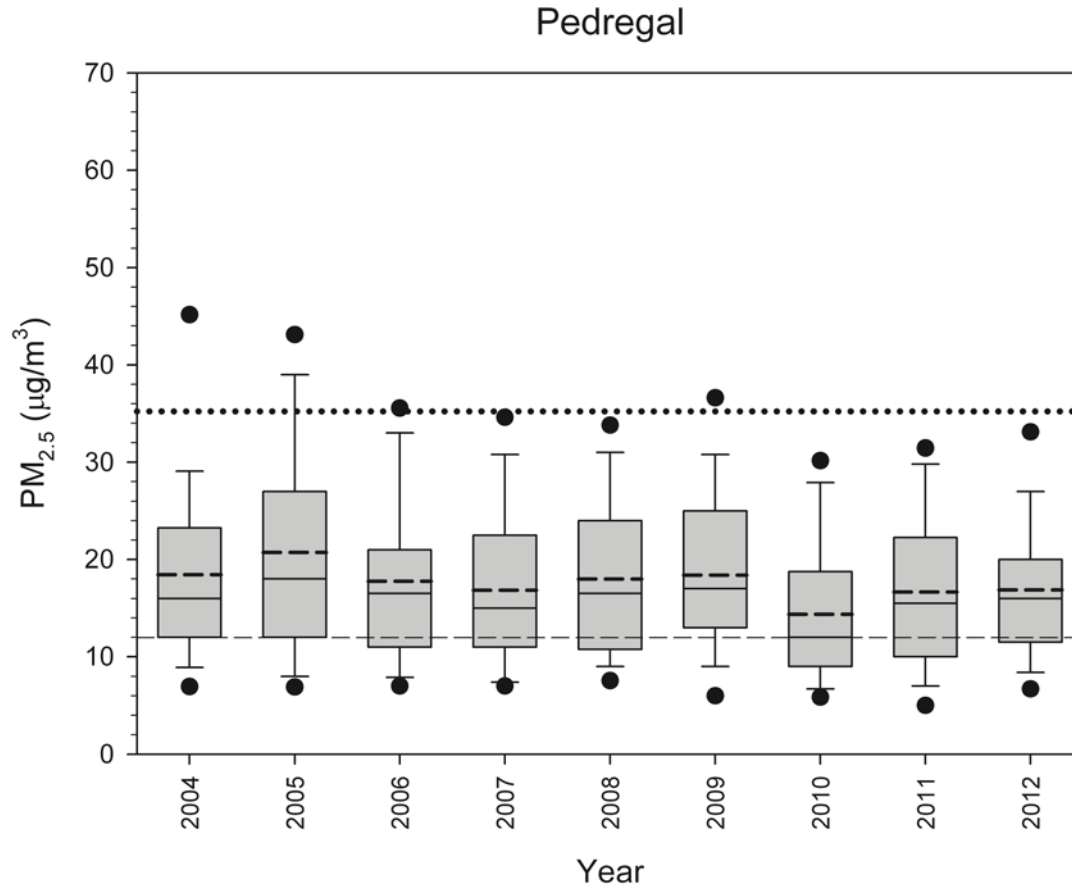
## FIGURES

Figure 1. Boxplots of the trend of PM<sub>2.5</sub> 24-hr average concentrations at Pedregal from 2004 through 2012. The dashed line and the continuous lines inside the boxes represent the annual mean and the median of the data, respectively. For comparison, the continuous gross dashed line and the thin dashed line inside the graph represent the PM<sub>2.5</sub> NAAQS concentration values for the 24-hour 98<sup>th</sup> percentile averaged over 3 years and the annual mean averaged over 3 years, respectively [52].

Figure 2. Boxplots of the trend of PM<sub>2.5</sub> 24-hr average concentrations at Xalostoc from 2004 through 2012. The dashed line and the continuous lines inside the boxes represent the annual mean and the median of the data, respectively. For comparison, the continuous gross dashed line and the thin dashed line inside the graph represent the PM<sub>2.5</sub> NAAQS concentration values for the 24-hour 98<sup>th</sup> percentile averaged over 3 years and the annual mean averaged over 3 years, respectively [52].

Figure 3. Boxplot trends of daily 8-hour mobile average O<sub>3</sub> concentrations at Tlalnepantla and Pedregal from 1989 through 2012. The gross and thin lines inside the boxes show the annual means and medians of the data, respectively. The dashed line inside the graphs represents the ozone NAAQS value which is based on the annual fourth-highest daily maximum 8-hr concentration, averaged over 3 years [52].

Figure 4. Two-way cluster analysis of the Pearson's correlation coefficients (PCCs) of the serum antibodies with environmental antibodies and metal concentrations. The figures on the left and right are for the Controls and Mexico City children respectively.



**Figure 1** Boxplots of the trend of PM<sub>2.5</sub> 24-hr average concentrations at Pedregal from 2004 through 2012. The dashed line and the continuous lines inside the boxes represent the annual mean and the median of the data, respectively. For comparison, the continuous gross dashed line and the thin dashed line inside the graph represent the PM<sub>2.5</sub> NAAQS concentration values for the 24-hour 98<sup>th</sup> percentile averaged over 3 years and the annual mean averaged over 3 years, respectively [52].

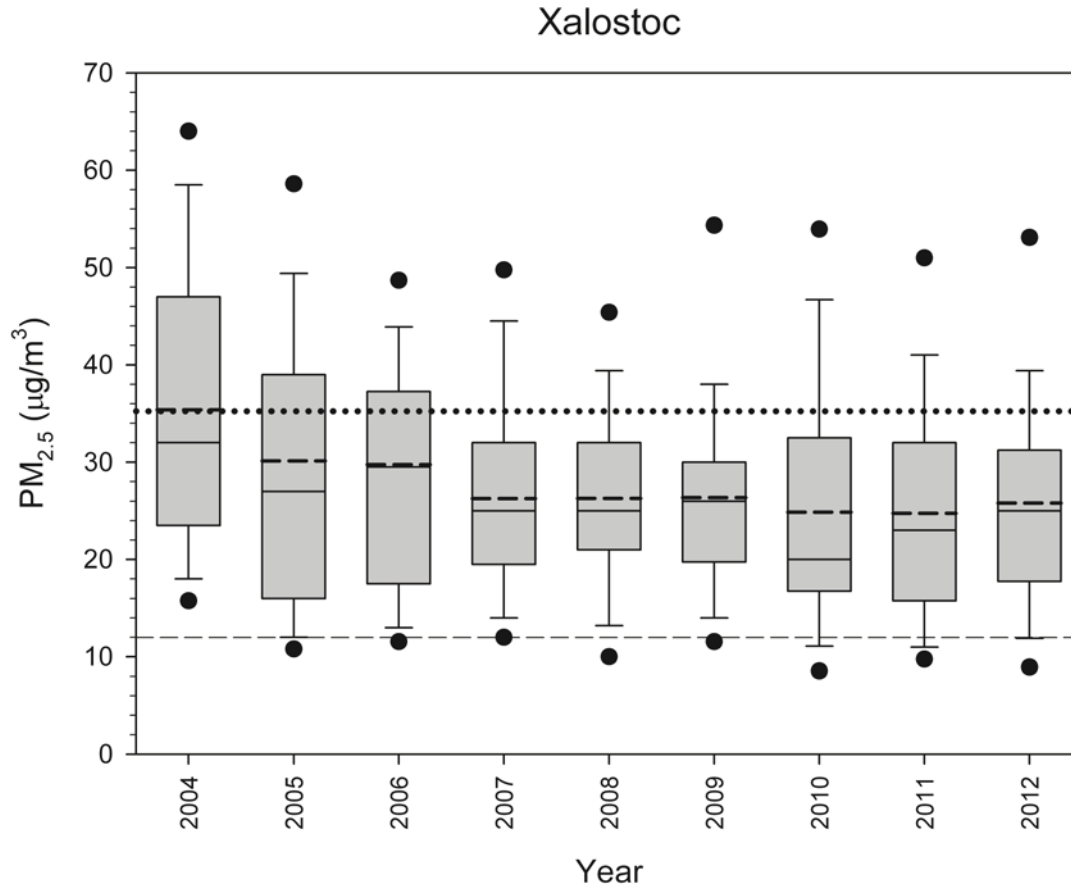


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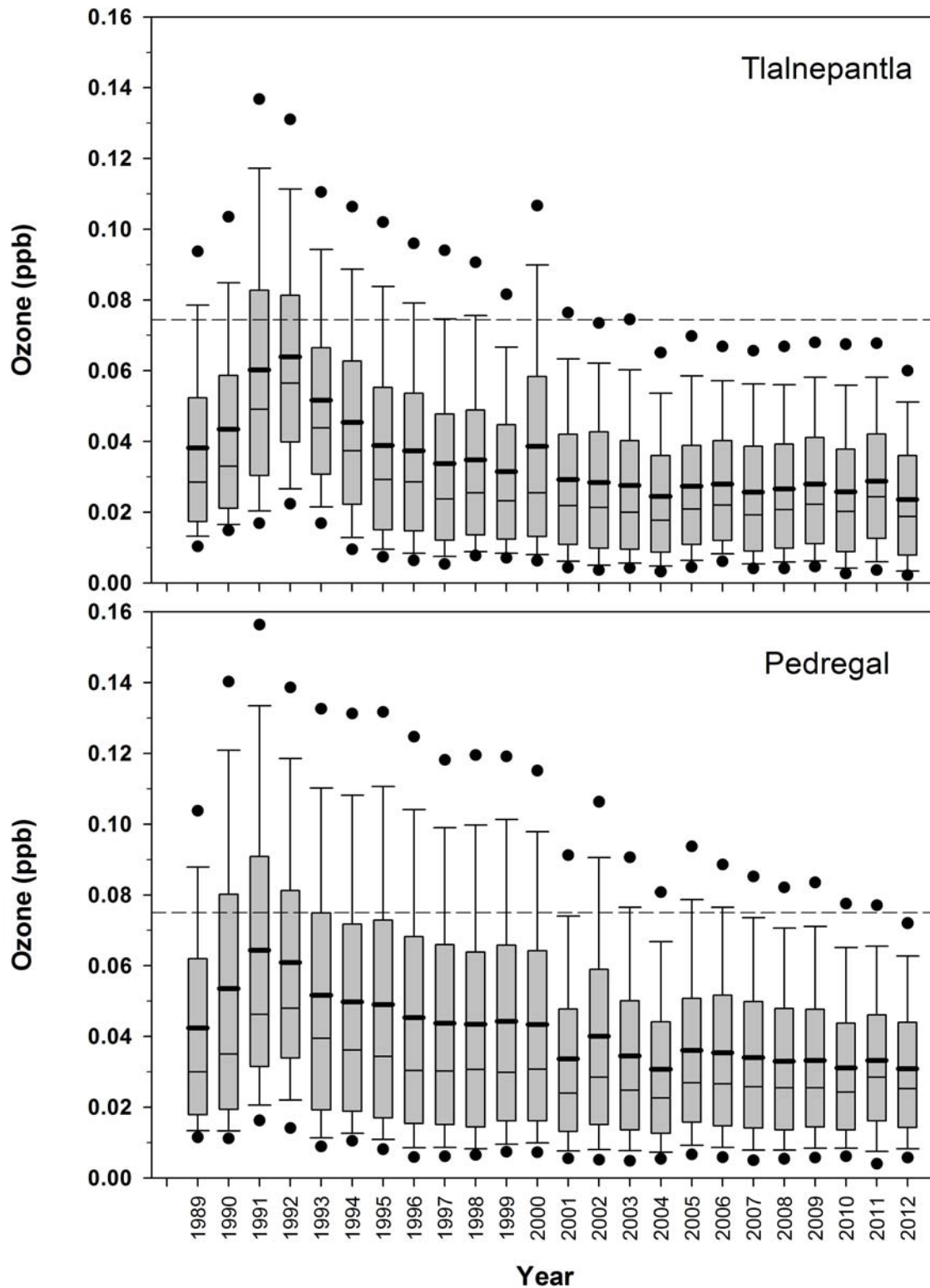


Figure 3. Boxplot trends of daily 8-hour mobile average  $O_3$  concentrations at Tlalnepantla and Pedregal from 1989 through 2012. The gross and thin lines inside the boxes show the annual means and medians of the data, respectively. The dashed line inside the graphs represents the ozone NAAQS value which is based on the annual fourth-highest daily maximum 8-hr concentration, averaged over 3 years [52].

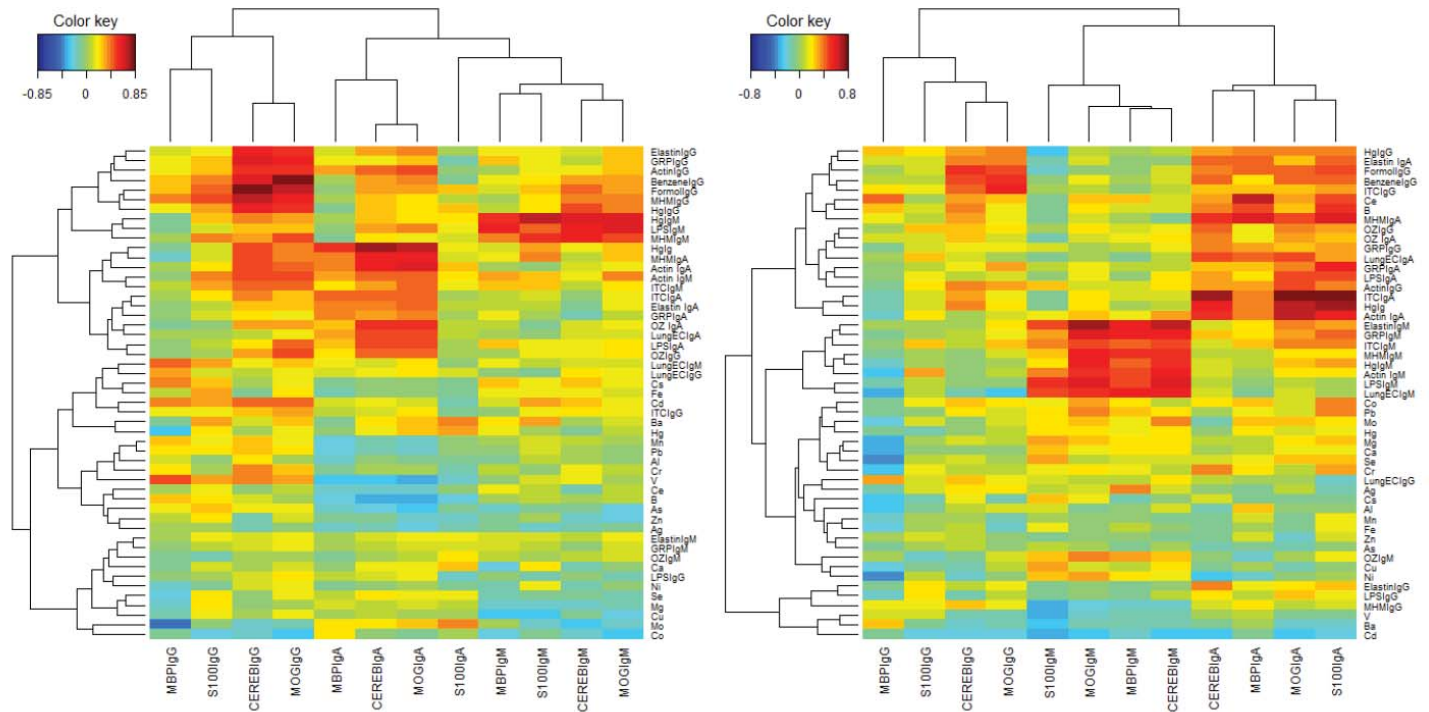


Figure 4. Two-way cluster analysis of the Pearson's correlation coefficients (PCCs) of the serum antibodies with environmental antibodies and metal concentrations. The figures on the left and right are for the Controls and Mexico City children respectively.

**Supplemental Table 1.** Metal isotopes and detection limits

Element	Isotope	Detection limits $\mu\text{g/L}$
Al	27	10.0
As	75	0.875
Ca	44	2500
Cd	111	0.500
Co	59	0.125
Cr	52	1.000
Cu	63	1.250
Fe	56	12.500
Hg	202	1.000
Mg	24	25.000
Mn	55	1.875
Ni	60	1.250
Pb	208	0.750
Se	78	12.500
V	51	0.125
Zn	66	25.000