

## The Intestinal Barrier in Air Pollution-Associated Neural Involvement in Mexico City Residents: Mind the Gut, the Evolution of a Changing Paradigm Relevant to Parkinson Disease Risk

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### Abstract

**Objective:** Braak et al proposal in 2003 “a putative environmental pathogen capable of passing the gastric epithelial lining might induce  $\alpha$ -synuclein misfolding and aggregation” could indeed be particulate matter gaining access through the most vulnerable section of the GI tract: the small bowel. This study is focused on the electron microscopy examination of tight junctions in duodenum of healthy dogs residing in one of the most polluted megacities in our continent, Mexico City Metropolitan Area (MCMA) with high concentrations of fine particulate matter (PM<sub>2.5</sub>) and nanosize PM versus low-air pollution controls and to measure serum antibodies to tight junctions (TJ) and neural proteins in MCMA versus low air pollution exposed children. The small intestine would be a prime PM target: it has a single unattached mucus layer, particles have easy access to epithelial cells and Peyer's patches, altering epithelial integrity and accessing the enteric nervous system. Autopsies in MCMA children v controls show extensive brainstem oxidative stress, microglial activation, and accumulation of  $\alpha$ -synuclein, from the dorsal motor nucleus of the vagus to the substantianigrae. Air pollution targets the dorsal vagal complex in mice exposed to the polluted MCMA atmosphere.

**Methods:** A pilot observational case-control dogs and children study of high versus low PM<sub>2.5</sub> exposures. We counted and evaluated the integrity of TJ's in duodenal electron micrographs from 6 MCMA dogs (5.01  $\pm$  1.36 years) and 4 control dogs (5.87  $\pm$  1.50 years) and we measured by ELISA serum antibodies to tight junctions (TJ) and neural proteins in 95 MCMA versus controls (11.02  $\pm$  3.6 years).

**Results:** Disruption of epithelial integrity with TJ structural changes in MCMA v control dogs (p<0.0001), the major determinant of paracellular permeability characterized the MCMA dogs' small bowel architecture. MCMA children had higher occludin-zonulin, actin, transglutaminase 3 and 6, and glutamic acid decarboxylase autoantibodies (p<0.01).

**Conclusion:** The integrity of the gastrointestinal (GI) barrier is significantly compromised in MCMA dogs and could be altered in MCMA children as evidenced by the autoimmune response to TJ and neural proteins. The GI breakdown likely impacts neuronal enteric populations and PM could reach the vagus and the brainstem. In the setting of urban air pollution, the evolution of a changing paradigm favoring a pathogen penetrating an epithelial lining and via trans-synaptic transmission reaching preganglionic parasympathetic motor neurons of the vagus nerve has to entertain particles as a potential culprit. Defining the linkage and the health consequences of the brain/ gut/ immune system interactions in urban children showing already the early hallmarks of Parkinson's disease ought to be of pressing importance for public health, may provide a fresh insight into Parkinson disease pathogenesis and open opportunities for pediatric neuroprotection.

**Keywords:** Autoimmunity; Children; Nanoparticles; Particulate matter; Swallow PM; GI barrier; Parkinson; Tight junction and neural autoantibodies

### Introduction

Mexico City Metropolitan Area (MCMA) children with no known risk factors for neurological or cognitive disorders exhibit cognition deficits, brain structural and volumetric changes and the neuropathological hallmarks of Alzheimer and Parkinson's diseases i.e., tau hyperphosphorylation with pre-tangles, amyloid beta 42 (A $\beta$ 42) plaques, and misfolded  $\alpha$ -synuclein olfactory bulb and brainstem accumulation [1-8]. Lewy neurites and/or punctate  $\alpha$ -synuclein deposits in the olfactory bulb, trigeminal thalamic tract, mesencephalic V, reticular and raphe nuclei, the glossopharyngeal-vagus complexes

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and lung and heart autonomic ganglia are seen in MCMA v control children as young as 11 years old [7]. We have also shown significant upregulation of COX2 in the right vagus and of CD14 in both right and left vagus of teens and young adults residing in MCMA v controls, suggesting the vagus nerve plays a role in the brainstem inflammation and neurodegeneration process [7,8]. Balb-c mice directly exposed to the polluted MCMA environment for 16 months v clear air controls developed significant inflammation involving the dorsal vagal complex (DVC), similar to mice given intraperitoneal endotoxins [9]. Moreover, MCMA-exposed mice displayed significant DVC imbalance in genes for antioxidant defenses, apoptosis, and neurodegeneration [9].

Extensive data in the literature support human and animal breakdown of the nasal/olfactory, blood-brain-barrier (BBB) and alveolar-capillary barriers and the expression of detrimental genes associated to urban air pollution [10-13]. Significant membrane structural changes to tight junction protein complexes and cilia and increased permeability of the lung/blood barrier are described in association with tobacco smog containing a combination of particulate matter (PM) and gases [14-15]. Moreover, recent research links inflammatory bowel diseases, changes in gut microbiome, and abdominal pain with air pollution [16-20]. The work by Kish and colleagues is of particular interest to us given the increased gut permeability in mice exposed to particulate matter with a diameter of  $<10\mu\text{m}$  ( $\text{PM}_{10}$ ) [18]. Braak et al. proposal [21] "a putative environmental pathogen capable of passing the gastric epithelial lining might induce  $\alpha$ -synuclein misfolding and aggregation" and the dual-hit hypothesis of Hawkes et al. [22] obligate us to think about the possibility swallowed particulate matter is gaining access to the brain through the most vulnerable section of the GI tract: the small bowel [23].

Very little is known regarding the ultrastructural features of the tight junction (TJs) in small bowel epithelium of young healthy animals with a lifetime exposure to air pollution. Likewise, the presence of immunological cascades in pediatric megacity residents with negative autoimmune histories, including celiac disease has not been explored. Given that MCMA children have lifetime exposures to high concentrations of PM and well documented breakdown of epithelial and endothelial barriers [2,7,10] and having the experience of studying healthy dogs exposed to the same environment as the children [1,24], we hypothesized that healthy MCMA animal facility dogs will have a breakdown of their small bowel cell junction integrity and children living in the same area will have significant higher levels of antibodies to host proteins involved in cell adhesion [25].

There were two primary aims of this study: 1. To explore by electron microscopy the integrity of the small bowel epithelium in healthy young dogs residents in MCMA and a control, low air pollution city, 2. To measure the serum concentrations of autoantibodies against barrier proteins from cohorts of matched age, gender, and socioeconomic status (SES) Mexican children with exposures to MCMA pollution versus low air pollution controls. Concomitantly, given the association between PM nanoparticle toxicity and the gastrointestinal route [18,20], we also explored transglutaminase 3 and 6, glutamic acid decarboxylase, and cerebellar antibodies.

Our results identify statistical significant abnormalities in the apical junctional complexes, desmosomal and gap junctions resulting in light and electron microscopy interepithelial gaps in the small bowel of MCMA dogs along with increased endocytic activity in the lamina propria endothelial cells loaded with nanosized PM. MCMA children versus controls showed significantly higher serum auto antibodies against occludin/zonulin associated with the integrity of tight junctions (TJs) and actin antibodies, a reliable marker of intestinal damage severity

in celiac disease and an indirect marker of histological and biochemical activity of autoimmune hepatitis [26-28]. MCMA children had higher titers of transglutaminase 3 and 6, and glutamic acid decarboxylase (GAD65) antibodies v controls. The presence of auto antibodies against key nervous system components carries a large impact for a brain in development, raising questions about their role in neuroinflammatory and neurodegenerative disease mechanisms [29]. Our study suggests that the integrity of the GI barrier could be compromised in highly exposed urbanites and short and long-term neural and extraneural health consequences including higher risk for Parkinson's disease have to be contemplated. Our ultimate intention is make the reader aware that the small bowel, a highly vulnerable section of the GI tract [23] could be key to explain our PD-like pathology and neuroinflammatory findings in highly exposed MCMA children, dogs and mice [4,6-9].

## Materials and Methods

### Study cities and air quality

Children's cohorts were selected from the Mexico City Metropolitan Area (MCMA) and small locations and cities in Mexico (Polotitlán, State of México; Zacatlán and Huachinango, Puebla; Zitácuaro, Michoacán; Puerto Escondido, Oaxaca; Chalma, Veracruz; Tlaxcala, Tlaxcala). The control cities have  $<75,000$  inhabitants and because of their small size it is expected that their levels for the main criteria air pollutants (ozone, particulate matter, sulfur dioxide, nitrogen oxides and carbon monoxide) could be lower than the current US EPA standards. Air quality monitoring in these locations is not common because they do not meet the minimum criteria of population and emissions for setting monitoring stations according the respective Mexican standard [30]. Our largest source of control children (40/47) 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 plant and one candle small factory. Polotitlán is included within the lower air pollutant emitters in the State of Mexico [31]. We have done extensive clinical studies in Polotitlán's healthy pediatric populations [1-5,10,29,32]. Because of the lack of historical continuous air pollution data in this location, we have followed the trend of its air quality based on the review of reports or unpublished data of both, emission inventories and measurements in the nearby region.

Mexico City Metropolitan Area is an example of extreme urban growth and accompanying environmental pollution [33-35]. The metropolitan area of over 2,000 km<sup>2</sup> lies in an elevated basin 2,200 m above sea level surrounded on three sides by mountain ridges. MCMA nearly 24 million inhabitants, over 50,000 industries, and 5.5 million vehicles consume more than 50 million liters of petroleum fuels per day, producing an estimated annual emission of 2.6 thousand tons of particulate and gaseous air pollutants [36]. MCMA motor vehicles produce abundant amounts of primary fine particulate matter ( $\text{PM}_{2.5}$ ). The high altitude and tropical climate where the MCMA is settled facilitate ozone production all year and contribute to the formation of  $\text{PM}_{2.5}$ . High ozone levels are typical of the warmer months (April to May) and PM higher levels are 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. Northern children have been exposed to higher concentrations of volatile and toxic organic compounds,  $\text{PM}_{10}$ , and  $\text{PM}_{2.5}$  including high levels of its constituents: organic and elemental carbon, nitro- and polycyclic aromatic hydrocarbons and metals (Zn, Cu, Pb, Ti, Mn, Sn,

V, Ba), while southern children have been exposed continuously to significant and prolonged concentrations of ozone, secondary aerosols ( $\text{NO}_3^-$ ) and particulate matter associated with lipopolysaccharide (LPS) [33-36]. Studies on the composition of  $\text{PM}_{2.5}$  with regards to sites and samples collected in 1997 show that composition has not changed during the last decade [33].

On the other hand, historical monitoring data in Polotitlán as well as mathematical modeling of air pollutants covering the central region of Mexico indicate that air quality in this part of the country has been typically below the equivalent US EPA air quality standards [37].

### Participants

This research was approved by the research ethics committee at the University of Montana and the Hospital Central Militar. Children gave active assent and their parents gave written informed consent to participation in the study. This work includes data from 95 children (49F, 46M) (Mean age=11.02 y, SD=3.6). 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. These children had a history of vaginal delivery, breast feeding for a minimum of 6 months and were introduced to solid foods after age 4 months. Mothers had unremarkable, full term pregnancies with uncomplicated vaginal deliveries and took no drugs, including alcohol. Children were examined by the attending pediatrician and considered clinically healthy. A pediatric nutritionist interviewed the child and asked the mother to keep a detail seven day food intake written record, including a weekend. Participants were from middle class families living in single-family homes with no indoor pets, used natural gas for cooking and kitchens were separated from the living and sleeping areas. All included children were actively engaged in outdoor activities and outdoor daily exposures in hours per day were recorded by the mother and child for 7 days, including the transit time to and from school, the time spent in recess and PE during school, the outdoor time while playing and engaging in other activities. Low (n: 47) and high (n:48) air pollution exposed children were matched by age, gender, socioeconomic status, and diets.

### Peripheral blood samples

Blood was collected between 7 am and 9.00 am 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  $-20^\circ\text{C}$  and then transferred to  $-80^\circ\text{C}$  and stored until further analysis.

### Reagents and methodology

**Proteins and Peptides:** Actin and tTG3 were purchased from Sigma/Aldrich St. Louis, MO. Various peptides HPLC grade with purity greater than 90% were synthesized by EZ Biolab Carmel, IN, including Glutamic Acid Decarboxylase (GAD-65), Transglutaminase-6, Occludin, Zonulin and Cerebellar peptide [38]. The procedure for the detection of antibodies by ELISA is described in detail in a previous paper [29].

### Dog small bowel samples

Previously harvested dog small bowel tissues for electron microscopy were used for this study. MCMA and control mixed beagles were whelped and housed in an outdoor-indoor kennel; husbandry was in compliance with the American Association of Laboratory Animal

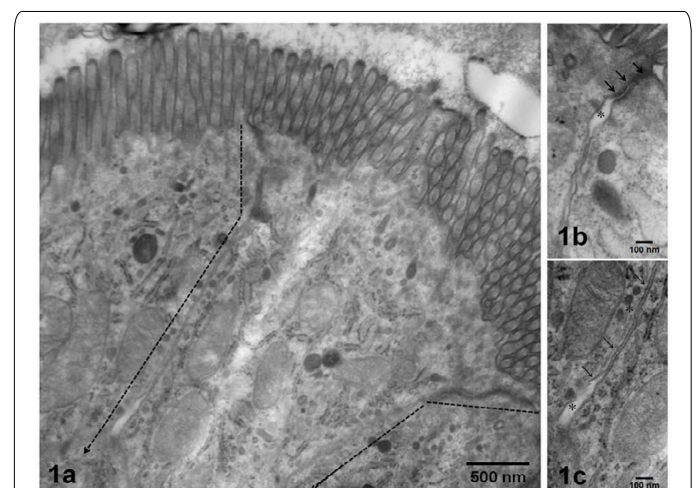
Certification Standards. Dogs were under daily veterinarian observation during their entire life, and at no time there was any evidence of respiratory, cardiovascular, gastrointestinal or neurological diseases. Dogs had all applicable vaccines and were treated with antihelmintics regularly. Dogs from both cohorts had the same diets. We selected to use small bowel optimally fixed electron microscopy tissues from 6 dogs ( $5.01 \pm 1.36$  years) from an independent longitudinal study involving the use of Nimesulide® in mixed beagle dogs. The 6 selected dogs for this study were in the non-treated Mexico City dog group exposed 24/7 to the Southwest MCMA atmosphere from birth. Four dogs average age  $5.87 \pm 1.50$  years from a low pollution control city were also studied. Procedures used were in accordance with the guidelines of the Use and Care of Laboratory Animals (NIH Pub No.86-23).

### Light microscopy

Sections  $1 \mu\text{m}$  thick were cut and stained with toluidine blue. Board-certified pathologists (LCG, ACG) without access to the identification codes reviewed the sections.

### Examination of small bowel samples by Transmission Electron Microscopy (TEM)

Tissues were post-fixed in 1% osmium tetroxide and embedded in Epon. Semi-thin sections ( $0.5$  to  $1 \mu\text{m}$ ) were cut and stained with toluidine blue for light microscopic examination. Ultra-thin sections ( $60$ - $90 \text{ nm}$ ) were cut and collected on slot grids previously covered with formvar membrane. Sections were stained with uranyl acetate and lead citrate, and examined with a JEM-1011 (Japan) microscope. Each electron micrograph was evaluated separately, and then compared by group. We captured ultrastructural epithelial images including sites of TJ's complexes. We evaluated 100 TJ's in each cohort. Electromicrographs were taken from TJ' complexes in epithelial cells starting at the apical region and continuing towards the base of the cell in a  $5000 \text{ nm}$  section (Figure 1). Electromicrographs were evaluated blindly and the number of abnormal TJ's counted (Figure 1).



**Figure 1:** Quantification and evaluation of the tight junctions were done in electron micrographs by counting all TJ's in epithelial duodenal cells starting at the apical region and continuing towards the base of the cell in a  $5000 \text{ nm}$  section (Control dog in Figure 1a, EM x 30,000 ). Electromicrographs were taken from each animal and the number of abnormal TJ's identified (Figures 1b, c). Figure 1B shows an abnormal zonula occludens (short arrows) along separation of the adjacent cytoplasmic membranes (\*) in an exposed dog (EM x 80,000). Figure 1C shows intact gap junctions (arrows) adjacent to areas with discontinuity of the junctions (\*) (EM x 80,000).



## Data analysis

For the antibody data, we first calculated the sample mean and sample standard deviation of each of the characteristic variables including the measurements of the antibodies 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. 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 0.05. Next, we separately calculated the percentages of the Mexico City children that showed measurements higher than the control mean and the control median for some selected variables. We also calculated the p-values of those percentages for testing how different they are from 50%. We calculated Pearson's correlation coefficients (PCC) among each pair

Year	Pedregal		Xalostoc	
	Mean	SD	Mean	SD
1997	21.6	16.6	71.3	34.1
1998	29.3	16.8	64.9	25.4
1999	24.4	9.2	71	26.6
2000	24.7	11.3	54.8	25.3
2001	23.6	10.1	41.1	17.2
2002	23.1	9.7	38	13.7
2003	23.4	11.3	41.8	14.4
2004	18.4	9.4	35.5	14.7
2005	20.9	11.5	30.4	17.1
2006	17.8	8.4	29.8	15.6
2007	16.2	8.5	25.3	11.3
2008	18	8.3	26.3	10
2009	18.4	8.7	26.4	10.7
2010	14.4	7.4	24.9	13.2
2011	16.7	8.3	24.7	11.5
2012	17	7.5	25.9	11.7

**Table 1:** PM<sub>2.5</sub> annual concentrations in µg/m<sup>3</sup> for North and South Metropolitan Mexico City area selected monitoring stations.

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. Abnormal TJ's identified by EM were counted for exposed and control dogs and p values calculated. We carried out the above mentioned statistical analyses in the statistical software 'R' (<http://www.r-project.org/>).

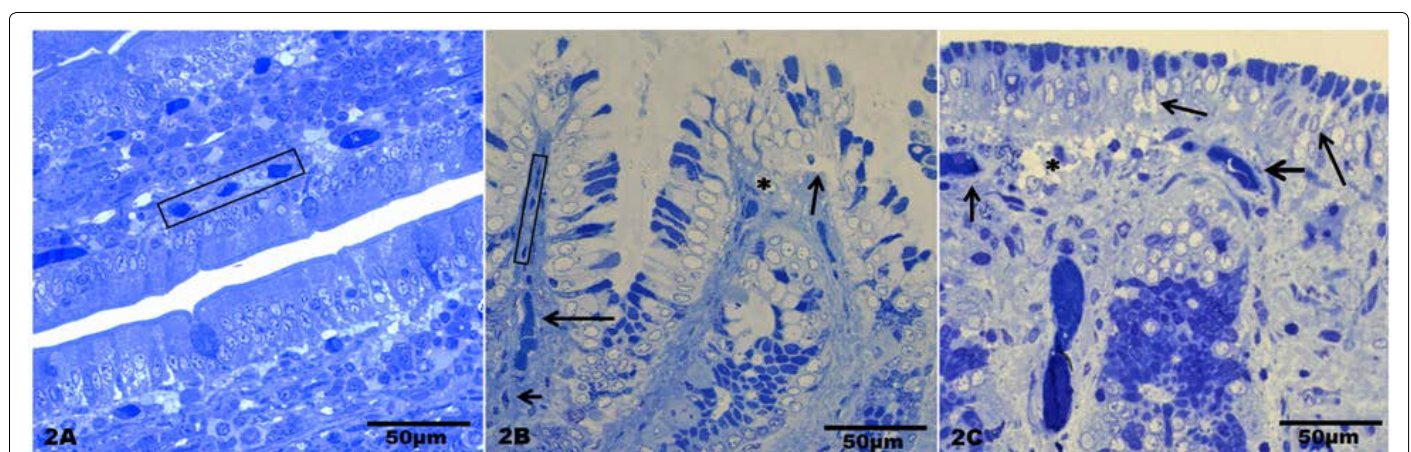
## Results

### Air quality data

Mexico City residents are exposed year-round to PM<sub>2.5</sub> concentrations above United States National Air Ambient Quality Standards (NAAQS). The PM<sub>2.5</sub> annual air quality standard of 12 µg/m<sup>3</sup> has been historically exceeded across the metropolitan area (Table 1). For this work we focused on particulate matter (PM), broadly defined by the diameter of the aerodynamic particles, and classified into coarse particles (<10 to >2.5 µm; PM<sub>10</sub>), fine particles (<2.5 µm, PM<sub>2.5</sub>) and ultrafine PM (UFPM<100 nm). Fine and ultrafine PM are of particular interest given their capability to reach the brain [39]. MCMA children in this study have been exposed to significant concentrations of PM<sub>2.5</sub> during their entire life, including the prenatal period. The high concentrations of PM<sub>2.5</sub> coincide with the time children play outdoors and/or stay in schools with broken windows and doors. All other criteria pollutants for MCMA, including nitrogen dioxide, sulfur dioxide and lead were at or 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.

### Electron Microscopic results

There was no statistical difference in the selected control v MCMA dogs' ages (p=0.35). Quantification of abnormal TJ's identified by EM yielded 14.2 ± 4.36 v 4.2 ± 1.3 in MCMA v controls (p<0.0001). Toluidine blue 1µm sections of normal duodenal epithelium with an intact brush zone and unremarkable enterocytes and goblet cells are characteristic of the control dogs (Figure 2A). In contrast, duodenum sections from Mexico City dogs exhibit segments of intestinal columnar



**Figure 2:** A. Toluidine blue 1µm thick section of a control duodenum in a 5 y old dog from Tlaxcala, a low-pollution city. The brush border is intact and there is an epithelium with unremarkable enterocytes and goblet cells. The lamina propria has unremarkable blood vessels (rectangular frame) and lacks inflammatory cells or degranulated mast cells. Toluidine blue x 40

B. MCMA 5.4 year old dog showed focal regions of the duodenal epithelium with a mild variation in the nuclear size of enterocytes and breakdown of the epithelial continuity (upper right arrow). There is significant submucosal tissue rarefaction (\*). The lamina propria has thickened wall vessels (rectangular frame). Arteriolar blood vessels with thickened walls (long arrow) surrounded by abnormal stroma (arrow head) and monuclear cells are observed. Toluidine blue 1µm thick section x 40

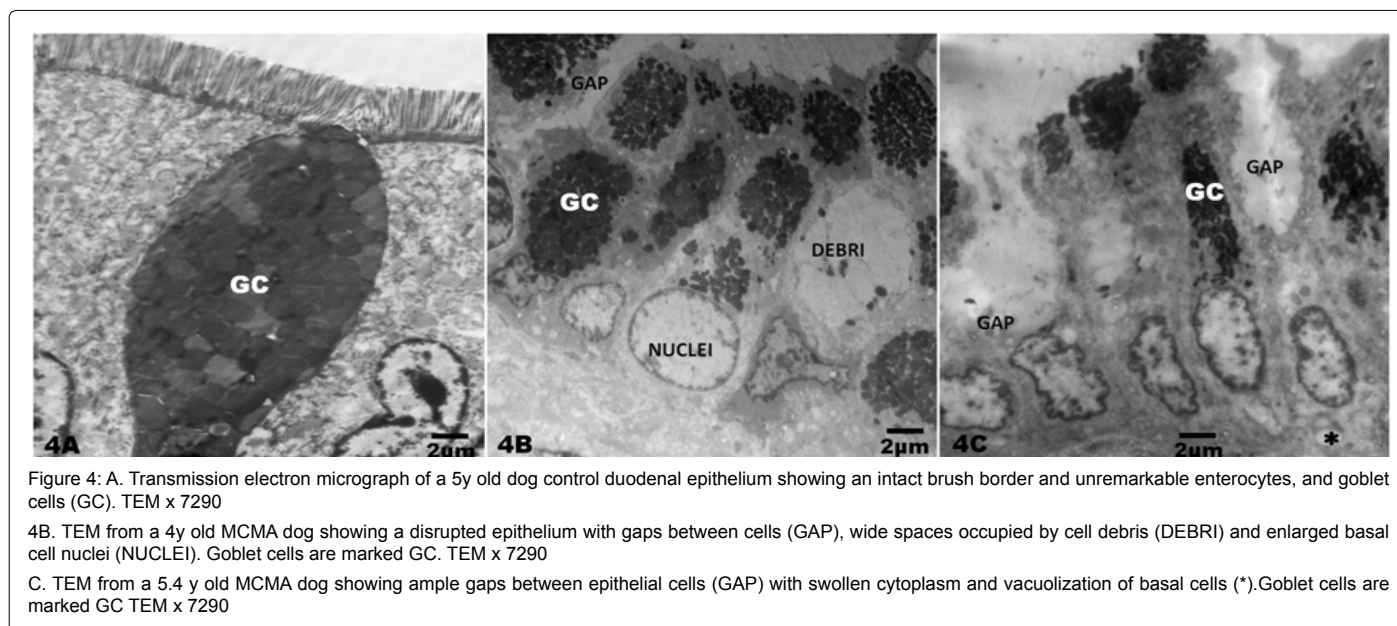
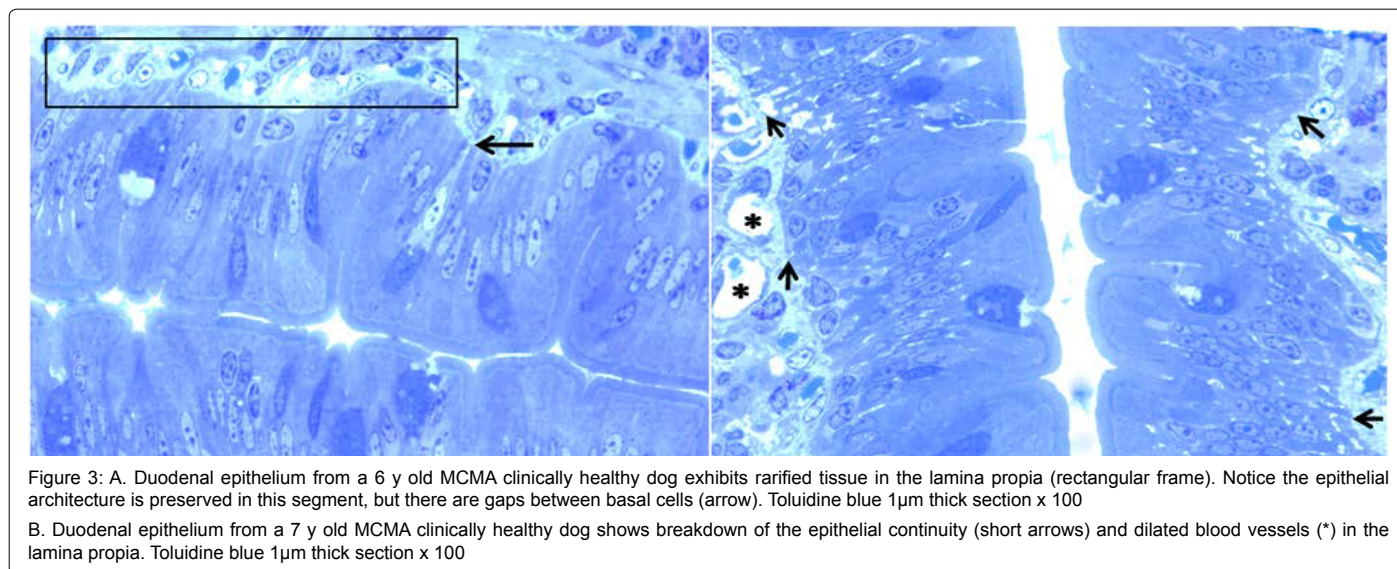
C. Duodenal epithelium from a 4y old MCMA clinically healthy dog shows a columnar epithelium with goblet cells alternating with enterocytes. The brush border is not visible. Focal regions of the epithelium exhibit breakdown of the epithelial continuity (long arrows). There is significant submucosal tissue rarefaction (\*). The lamina propria shows blood vessels with abnormal thickened walls (short arrows). Toluidine blue 1µm thick section x40.

epithelium with goblet cells and intercalated enterocytes with no visible brush border (Figure 2B). A few blood vessels in the lamina propria reveal thickened walls and focal disruption of the lamina propria (Figure 2B). Exposed dogs also display fibrosis at the level of the lamina propria (Figure 2C). Focal regions of the duodenal epithelium with a breakdown of the epithelial continuity, and submucosal tissue rarefaction with scattered mononuclear cells are observed in Figure 3A-3B. Low power transmission electron micrographs (TEM) reveal an intact epithelium and unremarkable brush border in a 5y old control dog (Figure 4A), while exposed dogs display a disrupted, fragmented epithelium with a few short villi in enterocytes, wide gaps separating epithelial cells, and intercellular spaces occupied by cellular debris (Figure 4B-4C). Control epithelium exhibits intact tight junctions and adherens junctions and regularly aligned microvilli (Figure 5A), while a wide range of abnormalities in TJ's can be seen in exposed duodenum (Figure 5B). Desmosomal complexes exhibited abnormalities in their intermediate filaments (Figure 6A). TJ's were significantly opened with

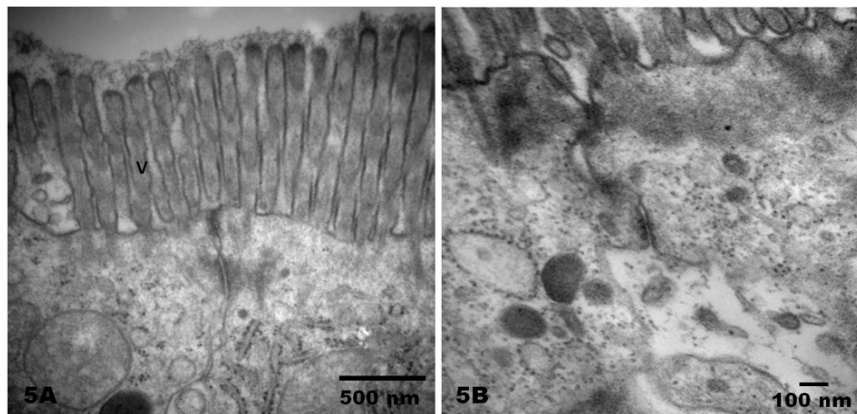
electron dense material occupying the junctional complexes (Figure 6B-6C). In Figure 7A, a control capillary with minimal caveolar activity and a red blood cell devoid of PM in a low pollution dog are seen, while in Figure 7B exposed endothelial cells exhibit numerous cytoplasmic clusters of nanosized particles in association with increased caveolae. An intraluminal red blood cell (RBC) displays numerous nanosized particles.

### Children's outdoor time, diet and serum results

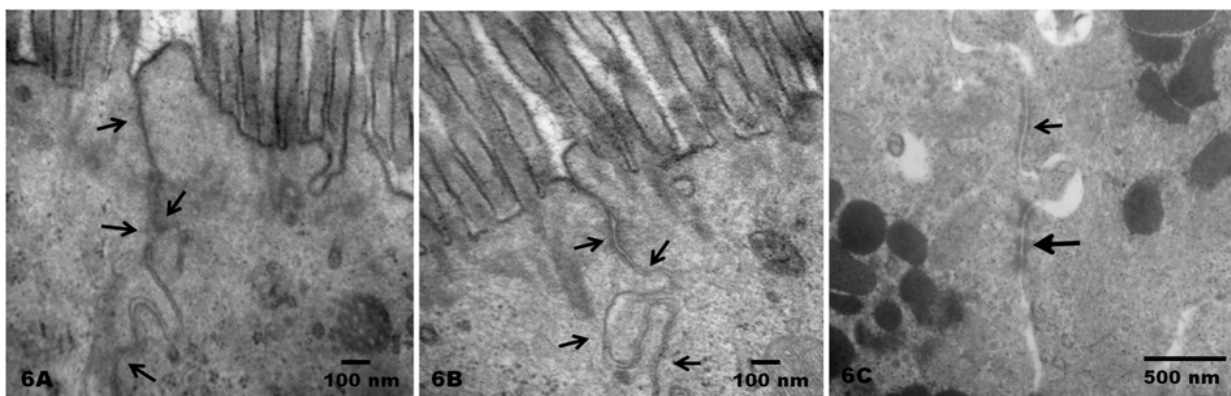
The number of hours spent outdoors was significantly different between groups, control children spent an average of  $5.19 \pm 0.91$  h v  $3.69 \pm 0.79$  h in the MCMA cohort ( $p < 0.0001$ ). The nutritional intake based on a seven day recall showed no differences in control v Mexico City children. When the intake of corn, wheat and rice products was analyzed, the control children had a significantly higher intake of corn ( $p = 0.009$ ) v MCMA, while wheat and rice showed no significant differences. The results of the 5 selected antigens from 47 controls and



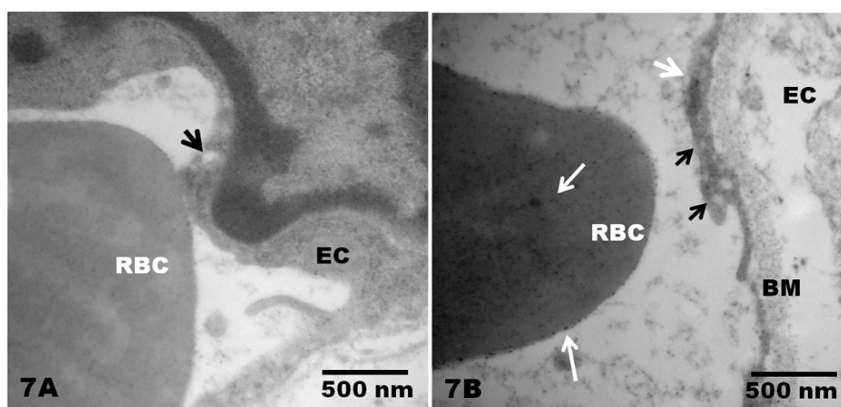




**Figure 5:** **A.** Control epithelium shows intact tight junctions, adherent junctions and regularly aligned microvilli (V) in the intestinal epithelium .TEM x 72,900  
**B.** In contrast, MCMA duodenal epithelium showing an upper TJ and lower desmosomal complexes with segmental discontinuity in their intermediate filament structure and a large gap in between cells. TEM x 117,000.



**Figure 6:** **A.** MCMA 5y old dog with an asymmetrical adherens junction structure, ill defined borders and accumulation of electron dense material (arrows) TEM x 117,000  
**B.** A four year old MCMA dog with zonula adherens junctions (arrows) showing focal discontinuity of electron dense material and intercellular widened spaces (>20nm). TEM x 117,000  
**C.** MCMA duodenal epithelium showing an upper TJ (short arrow) and lower desmosomal complexes (long arrow) with segmental discontinuity in their intermediate filament structure. TEM x 72,900.



**Figure 7:** **A.** Normal capillary with a red blood cell (RBC) devoid of PM in a low pollution exposed dog. A few caveolae are seen (short arrow) and there is no PM in the endothelial cell (EC) cytoplasm. TEM x 72,900  
**B.** MCMA 5.4 year old dog transmission electron micrograph of a blood vessel showing an endothelial cell (EC) with numerous cytoplasmic clusters of nanosized particles (short white arrow) on average 28 nm in association with increased caveolae (short black arrows). An intraluminal red blood cell (RBC) shows numerous nanosized particles (long white arrows). TEM x 72,900.

48 Mexico City children, age  $11.02 \pm 3.6$  years are shown in Table 2. The two selected cell junction antibodies: occludin-zonulin and actin showed significant statistical differences among cohorts for the high affinity IgG isotypes, while IgM was significantly increased for actin in Mexico City children. Transglutaminases 3 and 6, GAD 65 and cerebellar antibodies exhibited high IgG but not IgA isotypes for MCMA children and were significantly higher in exposed children. Table 3 shows the Pearson's correlation coefficients and p-values between the cell junction, and neural antibodies among Mexico City children. There was a striking correlation between cell junction and neural autoantibodies, particularly for actin and occludin-zonulin IgG isotypes. Age showed no significant correlations with any of the variables.

## Discussion

Disruption of epithelial integrity with structural changes in tight junctions (TJ), the major determinant of paracellular permeability, characterize the small bowel pathology in Metropolitan Mexico City healthy dogs. The integrity of the small bowel epithelial barrier is likely compromised as a result of the epithelial TJ disruption. These highly exposed dogs' intestinal findings could be relevant to the increases in actin and occludin/zonulin proteins autoantibodies seen in seemingly healthy MCMA children. We fully expect that disrupted GI barriers will allow for major concentrations of swallowed PM entering the GI tract, impacting neuronal enteric populations and reaching the vagus nerve. In this regard, PM gaining access through the most vulnerable

section of the GI tract: the small bowel, will be of deep interest to the possibility addressed by Braak et al. [21] and Hawkes et al. [22] about an environmental agent passing the GI epithelium and inducing abnormal changes in  $\alpha$ -synuclein. The presence of abnormal TJ's and brain autoantibodies in urban children with well documented systemic inflammation, neuroinflammation and the early brainstem and olfactory bulb hallmarks of Parkinson's disease obligates us to carefully look at the importance of the GI tract as a direct pathway to vulnerable brain regions.

The issue of the gastrointestinal route as a direct target and a simultaneous pathway for the entrance of air pollutant components through a disturbed barrier illustrated in Figure 8 is at the core of our concerns for exposed children. An immune-reactive response against barrier forming proteins is key to understand air pollutant mechanistic pathways affecting epithelial and endothelial barriers, including the GI barrier [10,18,40-43]. Ultrafine and fine PM resulting from combustion products are rich in organic and inorganic components, including heavy metals, benzene, formaldehyde, and endotoxins, and because of their pro-oxidative potential and their inflammatory capacity they increase the risk for toxicity [41]. Nanosized particles are capable to enter cells by an endocytic pathway, can penetrate through cells and through tissue resulting in cellular inflammatory reactions and toxicity [41]. Thus, in the scenario of sustained lifelong exposures to high levels of PM  $<2.5 \mu\text{m}$  and  $<100 \text{ nm}$  in diameter [33-35,44] and the capacity of PM to cause barrier damage, the presence of an impaired mucosal

	Control		Mexico City		Comparison p value	% above the control mean	% above the control median
	Mean	SD±	Mean	SD±			
OZ IgA	0.457	0.09	0.602	0.31	<b>0.003</b>	58.3	75
OZ IgG	0.482	0.15	0.581	0.16	<b>0.003</b>	62.5	68.8
OZ IgM	0.651	0.31	0.855	0.63	0.05	41.7	56.3
Actin IgA	0.596	0.19	0.679	0.26	0.08	54.2	62.5
Actin IgG	0.822	0.32	1.019	0.36	<b>0.006</b>	68.8	85.4
Actin IgM	0.724	0.22	0.873	0.35	<b>0.016</b>	68.8	68.8
TGM3 IgA	0.506	0.21	0.542	0.24	0.44	50	54.2
TGM3 IgG	0.659	0.22	0.825	0.3	<b>0.002</b>	68.8	70.8
TGM6 IgA	0.443	0.18	0.49	0.23	0.28	47.9	54.2
TGM6 IgG	0.589	0.17	0.736	0.37	<b>0.016</b>	58.3	66.7
GAD 65 IgA	0.406	0.14	0.452	0.17	0.15	50	66.7
GAD 65 IgG	0.578	0.17	0.671	0.2	<b>0.019</b>	66.7	72.9
GAD 65 IgM	0.58	0.18	0.674	0.28	0.05	62.5	62.5
CEREB IgA	0.511	0.17	0.617	0.34	0.06	41.7	54.2
CEREB IgG	0.545	0.23	0.845	0.44	<b>0.0001</b>	68.8	75
CEREB IgM	0.684	0.29	0.915	0.57	<b>0.015</b>	45.8	56.3

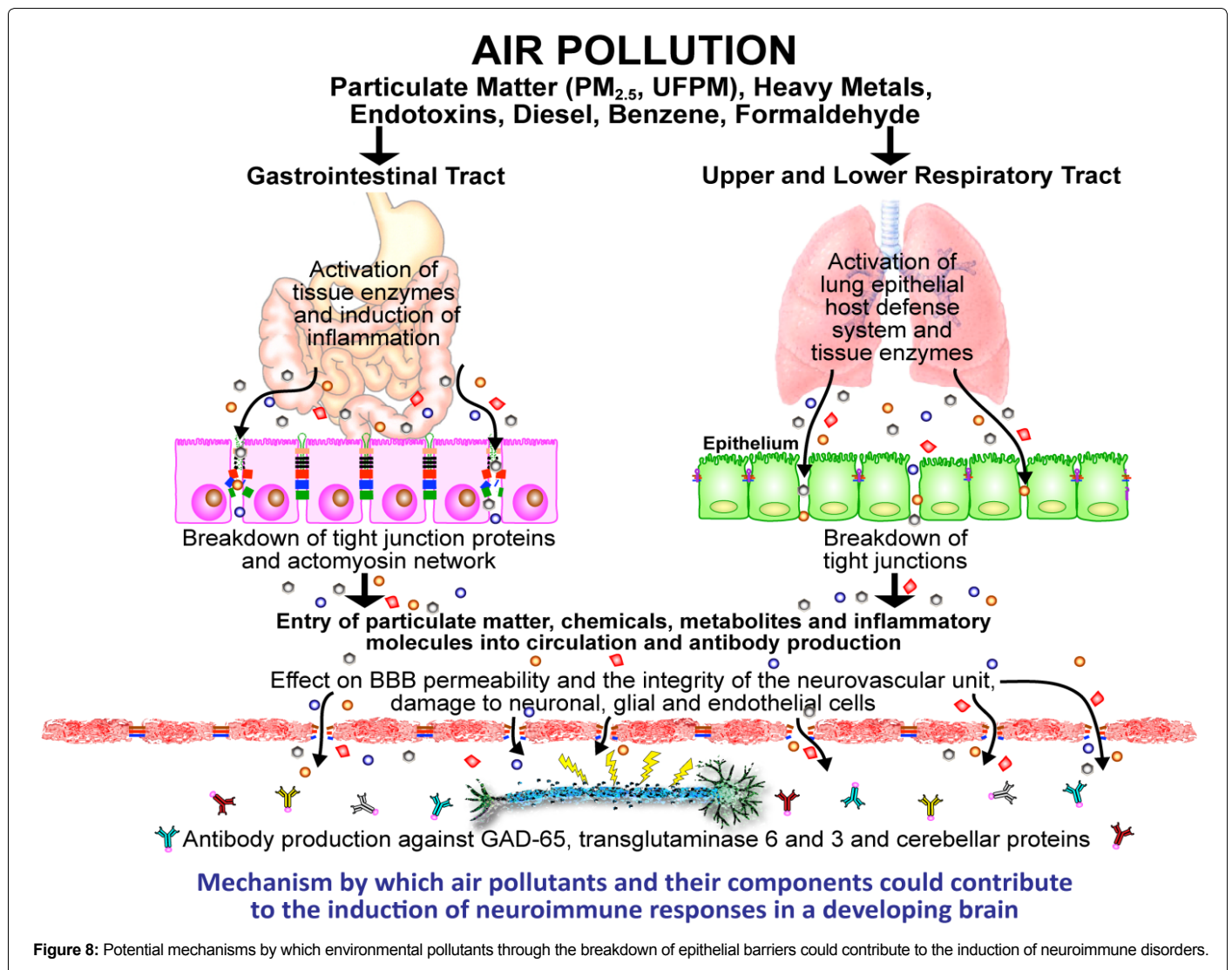
OZ=Occludin Zonulin, TGM3=Transglutaminase 3, TGM6=Transglutaminase 6, GAD-65=Glutamic Acid Decarboxylase, CEREB=Cerebellar peptide

**Table 2.** Serum autoantibodies in Controls v Mexico City Metropolitan Area children. Results are expressed in optical density units. Significant p-values are marked in bold. Percentages of the MCMA children that showed optical density values higher than the control mean and control median are also shown.

Autoantibodies and isotypes	GAD65 IgA	GAD65 IgG	GAD65 IgM	TGM3 IgA	TGM3 IgG	TGM6 IgA	TGM6 IgG	CEREB IgA	CEREB IgG	CEREB IgM
OZ IgA	0.22 (0.13)	0.13 (0.39)	0.28 (0.06)	0.20 (0.18)	0.28 (0.05)	<b>0.29 (0.04)</b>	<b>0.43 (&lt; 0.01)</b>	<b>0.40 (&lt; 0.01)</b>	0.26 (0.08)	0.22 (0.13)
OZ IgG	<b>0.44 (&lt; 0.01)</b>	<b>0.35 (0.01)</b>	0.28 (0.05)	<b>0.31 (0.03)</b>	<b>0.39 (&lt; 0.01)</b>	<b>0.43 (&lt; 0.01)</b>	<b>0.50 (&lt; 0.01)</b>	<b>0.43 (&lt; 0.01)</b>	0.27 (0.07)	0.16 (0.26)
OZ IgM	0.01 (0.93)	-0.16 (0.29)	0.31 (0.03)	0.13 (0.39)	0.15 (0.30)	0.08 (0.61)	0.11 (0.45)	-0.05 (0.76)	-0.08 (0.58)	0.28 (0.06)
Actin IgA	<b>0.72 (&lt; 0.01)</b>	<b>0.38 (&lt; 0.01)</b>	<b>0.48 (&lt; 0.01)</b>	<b>0.67 (&lt; 0.01)</b>	<b>0.57 (&lt; 0.01)</b>	<b>0.76 (&lt; 0.01)</b>	<b>0.64 (&lt; 0.01)</b>	<b>0.52 (&lt; 0.01)</b>	0.01 (0.95)	<b>0.31 (0.03)</b>
Actin IgG	<b>0.43 (&lt; 0.01)</b>	<b>0.54 (&lt; 0.01)</b>	<b>0.34 (0.02)</b>	<b>0.38 (&lt; 0.01)</b>	<b>0.38 (&lt; 0.01)</b>	<b>0.42 (&lt; 0.010)</b>	<b>0.43 (&lt; 0.01)</b>	<b>0.31 (0.03)</b>	<b>0.38 (&lt; 0.01)</b>	0.24 (0.11)
Actin IgM	<b>0.40 (&lt; 0.01)</b>	0.19 (0.20)	<b>0.76 (&lt; 0.01)</b>	0.24 (0.10)	0.25 (0.09)	<b>0.35 (&lt; 0.01)</b>	<b>0.34 (0.02)</b>	0.13 (0.38)	-0.05 (0.74)	<b>0.58 (&lt; 0.01)</b>

OZ=Occludin Zonulin, TGM3=Transglutaminase 3, TGM6= Transglutaminase 6, GAD-65=Glutamic Acid Decarboxylase, CEREB=Cerebellar peptide.

**Table 3.** Pearson's correlation coefficients between the neural and cell junction autoantibodies within the group of Mexico City children. The numbers within the parenthesis are the p-values for testing the significance of the corresponding Pearson's correlation coefficient. The significant ones are marked in bold.



barrier, and autoantibodies against barrier forming proteins are not unexpected findings in urban children.

Although we and others, have shown the nasal/olfactory, alveolar, endothelial and the BBB are compromised upon exposure to air pollutants [6-7,10,15-18,41] the GI barrier has been a less explored target. The GI tract is very important for several reasons: i.e direct ingestion of inhaled PM is common after being mobilized up the trachea via the mucociliary escalator and the particle size determines whether is cleared out by cough or swallowing [45], ii. Large increases in ventilation and GI intake of particles occur with increasing activity [46-48], a situation that is critical in children with outdoor physical activities in polluted environments. Once within the GI tract, PM enters in direct contact with luminal components, the mucus layer and the microbiome [20]. Very small particles in the nanosized range could gain direct access to the blood stream from the GI tract, while others damage the GI mucosa and alter the immune function. One critical issue to take into account is the vulnerability of the different GI anatomical compartments to PM. Indeed, in keeping with Johansson and colleagues [23] review of the GI mucus system; the small intestine would be the prime PM target: it has a single unattached mucus layer; nanoparticles can have easy access to epithelial cells and to Peyer's patches, affecting immunosurveillance

and altering epithelial integrity [49-52]. Compelling evidence shows that the small intestinal barrier function is immature in neonates, the anti-microbial peptide-dependent barrier function is weaker earlier in life [53] thus, the PM GI exposure-threat is present starting at birth for urban dwelling residents.

MCMA pre-adolescents and adolescents have a very low IgA but high levels of IgG against tTG-3, tTG-6, and GAD-65, which is an indication of autoimmune reactivity. The production of IgG isotype against various proteins (tTG-3, tTG-6, GAD-65) most likely relates to the detrimental impact of swallowed particulate matter upon the GI barrier, resulting in structural disruption and immune responses. These seemingly healthy children have not shown GI complaints and the increased burden of illness and use of health care pediatric services associated with celiac disease CD [54,55]. Our brain has very complex connections to the gut and there is bidirectional communication between GI cells and the CNS [56]. We agree with Hadjivassiliou et al. [57] that given TG6 is primarily expressed in the CNS, its presence obligates to question whether the intestine or the cerebellum primed the TG6 response in gluten ataxia. More importantly, TG6 is a protein associated with CNS development and motor control and an early brain insult and associated inflammation may predispose to future



development of TG6 autoimmunity [58,59]. Thus, the critical role of TG6 in cortical and cerebellar neurons is very relevant in the context of air pollution particularly because we previously reported the presence of finger to nose dysmetria, gait deviation and positive Romberg in MCMA children of similar age as this cohort [4].

Complicating the neural autoimmune scenario, Mexico City children also have high titers of antibodies to the enzyme glutamic acid decarboxylase (GAD65) associated with the presence of gait abnormalities and the Stiff person syndrome, type 1 diabetes, and anxiety disorders [60-63]. Autoantibodies to GAD interfere in vitro with GABA production and in vivo have negative effects in the entire CNS GABAergic system resulting in unbalance of excitatory and inhibitory neurotransmission [64-66]. The potential development of non-celiac gluten sensitivity in MCMA children is an interesting clinical issue. A plausible immunogenic pathway resulting in non-celiac gluten sensitivity includes fine and ultrafine PM causing damage to the TJ's at the most vulnerable follicle-associated epithelium protected by a single layer of epithelial cells and an easily removable mucus layer, [49] followed by an immune response and the production of TJ's antibodies. The breakdown of the barrier could be followed by gluten sensitivity and the formation of transglutaminases 3 and 6, GAD 65 and cerebellar antibodies. The issue of non-celiac gluten sensitivity (NCGS) in the setting of severe PM air pollution has to be entertained even when these children do not have CD symptoms [67,68].

A critical issue for pediatricians and parents alike should be: what is the clinical impact of transglutaminases 3 and 6, GAD 65 and cerebellar antibodies in urban children? Neural reactive antibodies are present in approximately 2-3% of the general population and most researchers will agree they do not usually contribute to CNS or PNS pathology [69]. However, it is becoming clear that neural antibodies can penetrate brain tissue either early in development or under pathological conditions (i.e., BBB damage) [70]. The association of neural autoantibodies and pathogenicity with a leaky BBB could be very important for highly exposed children [1,7] and as Levin and coworkers suggested a "defective BBB allows access of autoantibodies to targets on the brain cells" [70]. The major factor determining the impact of the brain autoantibodies is the integrity of the BBB, which in turn is determining the extent and degree of reactivity and detrimental brain responses [69-72].

The presence of nanosized PM in small bowel endothelial cells deserves a special comment. The endothelial cells showed accumulation of single particles and conglomerates measuring 14 to 55 nm [73]. Endothelial cells have a higher expression of caveolae in comparison with epithelial cells, and the charge and lipophilic characteristics of a nanoparticle surface play a key role in their cellular uptake [74]. These are interesting observations because small molecular weight antigens such as dextran and bacterial LPS enter the lamina propria via goblet cell associated passage ways [75]. Internalization of particulate antigens i.e., bacterial cell debris and nano PM co-localized with the CD11c+ dendritic cells in the lamina propria [75]. Intestinal epithelial cells nano PM uptake depends on their size: 20-40 nm NPs are taken up readily, while NPs larger than 100 nm are taken up mainly by the epithelial cells overlying Peyer's patches [75]. Thus, nano PM in GI endothelial cells in our study falls within the expected size range and we anticipate that the chemical composition of nano PM will have an impact on their cytotoxic effect.

Lastly, what is the impact of the GI tract as a portal of ultrafine (<100 nm) PM to the systemic circulation and the brain? Is this pathway

key for the development of the early stages of Parkinson's disease we are already documenting in children? [4,7,8]. The distribution of misfolded  $\alpha$  synuclein in MCMA children follows precisely the early stages I and II of Braak's PD staging [76,21]. Exposed children have olfaction deficits and their olfactory bulbs show misfolded  $\alpha$  synuclein [2]. Olfactory dysfunction precedes the onset of motor symptoms by years [77] and the intranasal administration of neurotoxicants in experimental animals supports the olfactory vector hypothesis of Parkinson's disease [78]. We have empirically followed children with severe orthostatic hypotension and syncope that could represent the most vulnerable children to PD non-motor early effects [8]. The role of the intestinal barrier integrity, the intestinal microbiome, the experimental evidence showing that different  $\alpha$ -synuclein forms can propagate from the gut to the brain, and the impact of molecular mimicry in the development of a disease for which there is no cure, can not be ignored [79-84].

## Looking Forward and Limitations

While recognizing that the evaluation of the TJ's was done in a small number of clinically healthy control and exposed dogs, and the serum antibodies in healthy control and exposed children, the results are nonetheless important to guide future investigations covering the gaps between the increase in TJ's and neural antibodies and the development of Parkinson's disease in highly exposed children. The significant damage to the TJ's is a major factor for compromising paracellular permeability and thus allowing the entrance of bacteria, viruses, toxic chemicals and especially nanosized particulate matter [41]. A direct impact on the small bowel immuno surveillance capacity is expected [50] and the children's autoimmune responses are critical given the formation of brain autoantibodies in the setting of a neurovascular unit that is equally compromised [69,85-86]. We fully agree with Levin and Diamond groups that in the presence of BBB compromise brain autoantibodies might contribute to initiation and/or pathogenesis of a wide spectrum of neurological diseases [69,70]. The issue is of critical importance in the developing brain because the damage may have short and long term detrimental consequences, including the development of Parkinson's disease [7,8]. Since we strongly argue that early dysregulated neuroinflammation results in misfolded key brain proteins (i.e.,  $\alpha$  synuclein) as a protective initial response, chronic exposure to air pollutants could result in hyperphysiological induction of highly stable fibrillar aggregates and neurodegenerative progressive events [87]. We fully agreed with Brandenberger et al. [88] that although transmission electron microscopy (TEM) is an appropriate technique to use for visualizing NPs inside cells, elemental analysis is recommended to confirm the presence of NPs inside the cell.

## Summary

Fine tuning of immune-to-brain communication is crucial to neural networks proper functioning, thus the evidence of GI barrier disruption associated with significant levels of cell junction and brain autoantibodies, is a subject of deep concern for urban children. There is increasing evidence linking diverse forms of air pollution to neuroinflammation and neuropathology seen in Parkinson's disease, including natural exposures of mice to the MCMA environment [9], elevated  $\alpha$  synuclein in midbrain of mice exposed to high concentrations of diesel exhaust particles (DEP) [89] and nanosize DEP activating microglia through multiple mechanisms and resulting in DEP-induced microglial  $H_2O_2$  production and loss of dopaminergic neuron function [90]. These animal studies and our neuropathology results in children and young adults strongly suggest that air pollution exposures may be associated with early Parkinson's disease-like pathology.

A large body of work on the GI immunosurveillance role, effects of disrupted TJs in the small bowel, goblet cell responses, small bowel mucus properties, GI immunization, brain developmental programming, autoimmunity, brain-reactive antibodies and disease already exists [25,49-52,69-70,91-97], thus expanding this knowledge in the scenario of air pollution pediatric GI effects could greatly facilitate our understanding of the downstream mechanisms of the complex interaction of antigens and neural targets and to elucidate if the GI cell junction lack of integrity are the culprit of pediatric urban diseases and carry a future risk for neurodegenerative fatal diseases. Also of utmost relevance is the importance of the gut microbial ecology and the contributions of non-pathogenic commensal flora to the development of CNS-autoimmunity [56,71].

Defining the linkage and the health consequences of the brain/gut/immune system interactions in children chronically exposed to air pollutants showing already the early hallmarks of Parkinson's disease ought to be of pressing importance for public health, may provide a fresh insight into Parkinson disease pathogenesis and open opportunities for pediatric neuroprotection.

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