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Alzheimer's disease and alpha-synuclein neuropathology in the olfactory bulbs of children and young adults ≤40years exposed to high levels of fine particulate matter air pollution in Metropolitan Mexico City.

APOE4 carriers at higher risk of suicide accelerate their olfactory bulb damage.

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ABSTRACT

There is growing evidence that air pollution is a risk factor for a number of neurodegenerative diseases, most notably Alzheimer's (AD) and Parkinson's (PD). It is generally assumed that the pathology of these diseases arises only later in life and commonly begins within olfactory eloquent pathways prior to the onset of the classical clinical symptoms. The present study demonstrates that chronic exposure to high levels of air pollution results in AD- and PD-related pathology within the olfactory bulbs of children and relatively young adults ranging in age from 11 months to 40 years. The olfactory bulbs (OBs) of 179 residents of highly polluted Metropolitan Mexico City were evaluated for AD- and alpha-synuclein-related pathology. Even in toddlers, hyperphosphorilated tau (hTau) and Lewy neurites (LN) were identified in the olfactory bulbs. By the second decade, 86% of the bulbs exhibited hTau (50/57), 76% LNs (45/57), 77% vascular amyloid (44/57), and 60% (34/57) mild diffuse amyloid plaques. During the first two decades, OBs neurovasculature unit damage is associated with combustion-derived nanoparticles and myelinated and unmyelinated axonal damage was evident. OB hTau neurites were associated with pretangle stages 1a and 1b in subjects ≤20 years of age, strongly suggesting olfactory deficits could potentially be an early guide of AD hTau stages. Compared to non-carriers, APOE4 carriers were 6 to 13 times more likely to exhibit OB vascular amyloid, neuronal amyloid accumulation, alpha-synuclein aggregates, hTau neurofibrillary tangles, and neurites. Remarkably, within this data set the APOE4 carriers were 4.7 times more likely than non-carriers to have committed suicide. The present findings, along with previous evidence that over a third of clinically healthy teens and young residents from the targeted pollution areas exhibit low scores on an odor identification test, support the

concept that olfactory testing may aid in identifying young persons at high risk for neurodegenerative disease. Neuroprotective interventions in air pollution exposed individuals in the first two decades are critical. Air pollution control should be prioritized.

Keywords:

Alzheimer, alpha synuclein, alpha-synucleinopathies, amyloid plaques, air pollution, APOE4, children, corpora amylacea, combustion-derived nanoparticles CDNPs, hyperphosphorilated tau, Mexico City, olfactory bulb, Parkinson, PM $_{2.5}$, suicide, tauopathies, young adults.

Introduction

Exposure to air pollutants plays a major role in the development and/or acceleration of Alzheimer's disease (AD).[1-8] Highly exposed Metropolitan Mexico City (MMC) residents show an early brain imbalance in genes involved in oxidative stress, inflammation, and innate and adaptive immune responses.[9] Dysregulated neuroinflammation, diffuse brain neurovascular unit damage, the accumulation of misfolded proteins associated to the early stages of both Alzheimer's and Parkinson's diseases are seen in MMC youth and are absent in clean air controls. [2,3, 9-13]

Most of the literature associating air pollution to neurodegeneration is focused on Alzheimer's disease, with a few papers making an association with Parkinson's disease (PD).[14-19] We have described both, AD and PD neuropathological hallmarks in young

MMC residents, including hyperphosphorilated tau and beta amyloid plaques in children as young as 11 months, [20] and alpha-synuclein in key brainstem nuclei and tracts.[2, 10,12,13,21]

In a canine study comparing MMC dogs age 7 days to 10 years (n:26) versus clean air controls (n:14), nasal respiratory and olfactory epithelium were found to be early pollutant targets.[22] Olfactory bulb and hippocampal apurinic/apyrimidinic (AP) sites in nasal and brain genomic DNA, were significantly higher in exposed v control age matched dogs. [22] We previously described the olfactory bulb (OB) pathology in a cohort of 35 MMC vs 9 controls ages 20.8+/-8.5 years assessed by light and electron microscopy.[10] MMC residents showed with no exceptions OB vascular changes, neuronal accumulation of particles, and/or immunoreactivity (IR) to beta amyloid (29/35) and/or alpha-synuclein (4/35) in neurons, glial cells and/or blood vessels. [10] Combustion-derived nanoparticles (CDNPs) were documented in OBs endothelial cytoplasm and basement membranes. Control OBs were unremarkable. In the same work we also described the results of the University of Pennsylvania Smell Identification Test (UPSIT) administered to 62 MMC v 25 controls age 21.2+/-2.7 years. Olfaction deficits were present in 35.5% MMC and 12% of controls.[10] Of critical importance for the present work was the observation APOE 4 carriers failed 2.4+/-0.54 items in the 10-item smell identification scale from the UPSIT related to Alzheimer's disease, while APOE 2/3 and 3/3 subjects failed 1.36+/-0.16 items, a highly significant result p=0.01. [10]

The OB is a perfect environmental target, participates in the brisk neuroinflammatory process upon exposures to polluted air where particulate matter and metals are key components, along with endotoxins and CDNPs. [5,10,22,24-30] We have described the association between olfactory bulb apurinic/apyrimidinic (AP) sites in genomic DNA- the

most frequently formed DNA lesions in the genome, evidencing contact with DNA-damaging agents-, the presence of metals like Ni and V (from industrial environmental sources) in a gradient from olfactory mucosa > olfactory bulb > frontal cortex and the significant OB neuroinflammation with upregulation of IL1β and COX2 in MMC residents.[22, 30] Moreover, the clinical counterpart is seen in MMC children (13.4 ± 4.8 years, 28 APOE 3 and 22 APOE 4). APOE4 children strongly failed to identify the soap odor in the UPSIT, strongly correlating with left hippocampus higher mI/Cr ratio. [31]

Urban polluted environments and occupational exposures with ubiquitous high concentrations of ultrafine particulate matter (UFPM, nanosize particles < 100 nm) are of great concern for the nervous system due to the ease with which they penetrate biological barriers, including vascular endothelium, alveolar-capillary, olfactory, nasal, gastrointestinal, blood-brain-barrier (BBB) and blood-CSF barrier.[3,5,11-13,20]

Combustion-derived nanoparticles (CDNPs) are composed of iron and associated transition metals, are highly oxidative and strongly magnetic.[5] Such particles gain entry to the brain in significant amounts in young and older adult MMC residents and are known to cause severe damage to critical cellular organelles in the CNS in young urbanites.[3, 5, 11, 13,20]

We have one primary aim for this study: To document in young urbanites by immunohistochemistry the early stages of the olfactory bulb pathological process associated with Alzheimer and alpha-synucleinopathies. Electron microscopy is focused on the documentation of the vascular pathology, the identification and measurement of combustion-derived nanoparticles and the associated organelle pathology. We are

concerned about the olfactory bulb pathology progression with age and cumulative exposures to fine particulate matter ($PM_{2.5}$) above the USEPA standard.

The early identification of olfactory AD and alpha-synucleinopathies pathology in air pollution highly exposed young individuals and the understanding the mechanistic pathways involved in the development of fatal neurodegenerative diseases are at the core of our research efforts. Identifying key air pollutants impacting early neural risk olfactory trajectories would greatly facilitate multidisciplinary prevention efforts for modifying the course of AD and alpha-synucleinopathies in pediatric and young adult ages.

Methods

Study design and samples

One hundred and seventy-nine consecutive autopsies with sudden causes of death, not involving the brain were selected for this study. MMC subjects age 11 months to 40 years were clinically healthy prior to their sudden demise and were included in the study if:

i. sections of olfactory bulb contain the anterior olfactory nucleus, granular, plexiform and glomerular layers and olfactory tract white matter, ii. gross examination of the brain was unremarkable, and iii. macro and microscopic examination of extra-neural key organs was unremarkable. Examination of autopsy materials was approved by the Forensic Institute in Mexico City. Autopsies were performed 4.1 ± 1.7 h after death between 2004-2008, and samples were collected by 4 trained researchers, weekdays, weekends and holidays during the 5 year study period. Brains were examined macroscopically, sections were selected for light and electron microscopy, and frozen tissues collected. The general characteristics of the study population, including their cause of death are seen in Table 1 (Suppl). An average

of 46±11 olfactory bulb slides were examined per case. Paraffin embedded tissue was sectioned at a thickness of 7 μm and stained with hematoxylin and eosin (HE). Immunohistochemistry (IHC) was performed on serial sections as previously described.[2] Antibodies included: β amyloid 17-24, 4G8 (Covance, Emeryville, CA 1: 1500), PHF-tau8 (Innogenetics, Belgium, AT-8 1:1000), and α-synuclein phosphorylated at Ser-129, LB509 (In Vitrogen, Carlsbad,CA 1:1000). Brain tissues included in this work were previously blindly investigated for purposes of AD. [20] Olfactory bulbs were examined for AD and alpha-synucleinopathies hallmarks.[32-47] Tau pathology was scored using separate semiquantitative scores for neuropil threads (NTs) and neurofibrillary tangles (NFTs): 0=absent, 0.5=very mild(only single lesions), 1= mild, 2= moderate, and 3= severe.[36,38,39] The β- amyloid scoring was semiquantitative: 0=absent, 1= mild, few diffuse Aβ positive areas, no plaques, 2= moderate, ≤3 plaques HPFx200, and 3= severe, 4 or more plaques HPFx200. [38] Intracellular Aβ was scored 0=absent and 1= positive; vascular β- amyloid 0,1,2 and 3 (severe). Alpha-synuclein was scored as follows: 0=absent, 1= mild, few Lewy neurites, no Lewy bodies 2= moderate, more than 1 Lewy body in a low power field, and sparce LNs; 3= severe, >1LBs and scattered LNs LPF and 4 very severe, numerous LBs and LNs. [40] Olfactory bulb tissue blocks were processed for EM [3] with a focus on the neurovascular unit and the target organelles of CDNPs. Genotyping for the presence of APOE alleles polymorphisms was done as previously described.[3]

Air Quality Data

Metropolitan Mexico City residents are exposed year-round to fine particulate matter ($PM_{2.5}$) and ozone (O_3) concentrations above the United States National Air Ambient Quality Standards (NAAQS). For this study, we focused on <2.5μm particles and work

with cumulative $PM_{2.5}$ ($CPM_{2.5}$) above the annual USEPA standard: 12 μg/m^3, reflecting lifetime exposures above the standard. Both, the $PM_{2.5}$ annual standard and the 24-hr 35 μg/m^3 standard have been historically exceeded across the metropolitan area for the last 20 years.[23-25]

The accumulated burden of $PM_{2.5}$ for each subject-including pregnancy-was calculated based on their urban residency. Historical $PM_{2.5}$ levels were obtained from a combination of particulate matter (PM) data from Mexico City Government Manual Monitoring Network for five representative urban sites: Tlalnepantla (NW), Xalostoc (NE), Pedregal (SW), Iztapalapa (SE) and Merced (downtown) (Figure 1) and an approach considering the typical $PM_{2.5}/PM_{10}$ ratio for each of the representative sites. Historically, the highest $PM_{2.5}$ concentrations occur in the NE sector where industrial and traffic activities are prevalent and decrease towards the SW residential area. We selected to work with a cumulative $PM_{2.5}$ ($CPM_{2.5}$) exposure based on the assumption that long-term concentrations above the annual, averaged over 3 years USEPA standard of 12 μg/m^3, would have detrimental health effects. To estimate the backward $CPM_{2.5}$ we used the expression:

$$C\,PM_{2.5} = \sum \max\left(\left[\text{annual mean } PM_{2.5}\right] - 12\ \mu g/m^3, 0\right)\Delta t$$

where the "max" function ensures only annual mean $PM_{2.5}$ values are included. The backward summation was taken over the life time age (Δt) of each subject back to the prenatal period. The procedure was based on the assumption that the trend of $PM_{2.5}/PM_{10}$ ratio obtained from the slopes of the correlations of these species in the period 2004-2010 represent the backward $PM_{2.5}/PM_{10}$ ratios trends for previous years. The results compared well with a number of $PM_{2.5}/PM_{10}$ ratios reported by academic groups in conference

proceedings and published papers related to PM pollution in Mexico City in the 1980-1990s.[24-25] The resulting ratios were used to estimate the $PM_{2.5}$ annual averages for each of the selected sites for the period 1989 to 2003. Since the study population included individuals older than 30 years at their time of death, we assumed a constant value for the $PM_{2.5}$ annual averages prior to 1989 equal to the annual mean for this year. Overall, the $PM_{2.5}/PM_{10}$ ratios were relatively constant ranging from ~ 0.45 in the Southwest towards ~ 0.25 in the Northeast. High $PM_{2.5}/PM_{10}$ ratios indicate a dominance of coarse particles in the PM_{10} while low ratios are associated to prevalence of fine particles.

With the estimated $PM_{2.5}$ annual averages for each site and year, we obtained a working annual average by averaging the 3 previous consecutive years according with the procedure to calculate the USEPA annual mean standard, moving backwards in time up to 30 years. The resulting working annual average was used to obtain the $CPM_{2.5}$ with our equation for each of the individuals in the study, starting 1 year before their year of birth and up to the age of death (Table 1, Suppl). The working average data base was chosen according with the closest sampling site to their residence addresses during most of their life.

Chemical PM composition studies in Mexico City have shown that the proportion of the different component PM species has not changed significantly along the years. [24-29] The $PM_{2.5}/PM_{10}$ ratio variations and the PM chemical composition are dependent on the site location and on the season. Typically, the coarse PM in MMC is strongly dominated by geological material ($SiO_2+CO_2^{-3}+Al_2O_3+Ca+Fe+Mg+K$) from dust resuspension. Organic and carbonaceous aerosols are the dominant species in the PM fine fraction. Particle emissions from gasoline and Liquefied Petroleum Gas Combustion (LPG) are dominated by organic carbonaceous aerosols (OC), while in diesel particles, black carbon (BC) is the

main component. [26] Organic aerosols in the air include primary hydrocarbon-like compounds, oxygenated organic compounds mostly secondary, organics from biomass burning, and small contributions of nitrogen-containing organics of primary combustion.[27] Also, critical for the brain effects, BC concentrations in $PM_{2.5}$ have not shown a decrease through the years. [27] BC is associated with polycyclic aromatic compounds (PAHs), semi-volatile species resulting from incomplete combustion of carbonaceous fuels such as gasoline and diesel vehicle exhaust gases.[28] Most of PAHs in MMC are present in $PM_{2.5}$. Trace metals in fine particles include Zn, Cu, Pb, Ti, Sn, Ba, Mn, Sb, V, Se, As, Ni, Cd, Cr in that order. [29] Zn, Cu, Ba, Pb, and Cd are tracers of road traffic, while V and Ni are tracers of industrial emissions. Exposures to ozone (O_3) concentrations are also above the USEPA standards (annual fourth-highest daily maximum 8-hour concentration, averaged over 3 years) all year long (Figure 1). All other criteria pollutants for MMC, including nitrogen dioxide, sulfur dioxide and lead have shown elevated levels prior to 2000, but have been at or below the current EPA standards in the last 17 years.

Statistical analysis

Our sample size of 179 subjects was taken from a prior study of 203 subjects[20] and it was defined a priori by sampling logistics in the 5 year study period balancing the expected results from previous neuropathology studies in young urbanites.[2, 3, 12-13] We focused on summary statistics and graphical summary of the concerned staging variables: the major markers of Alzheimer and alpha-synucleinopathies (hTau, amyloid-β, α synuclein), age, gender, mode of death, and APOE status. Mode of death was analyzed in three major groups: accidents, homicides, and suicides. Subjects were divided by decades 1 and 2 (n: 57) and 3 and 4 (n: 122). Figure 2 shows the comparison between the percentages of hTau

and amyloid-β phases in brain [20] versus olfactory bulb hTau NTs, NFTs, alpha-synuclein, Aβ in OB, vascular amyloid and intraneuronal amyloid.[36,38-41-47] We also tested the relationship of the probability of committing suicide with respect to APOE status after adjusting age and CPM2.5 exposures. A goodness of fit test to check if the percentages of APOE4 in the suicide group and accident and homicide groups were the same. We identified APOE 4 carriers as individuals with higher suicide risk, and we performed logistic regression analysis to check if APOE 4 carriers had higher involvement of the targeted AD and alpha-synucleinopathies markers in the OB. We performed the statistical analyses using Excel and the statistical software 'R' (http://www.r-project.org/).

Results

Figure 1 and Table 1 (Suppl) show the annual mean averages of $CPM_{2\cdot5}$ for each individual based on their residence within arbitrary centroids in each of the five selected sampling sites. A polynomial regression of second degree was applied to the $CPM_{2.5}$ data for each site. The regressions were overlapped on a figure of the estimated spatial distribution of the annual average $PM_{2\cdot5}$ concentrations in MMC for 2008. The insert in figure 1 shows the annual average of the daily ozone 8-h maximum for the same year.

Figure 2 shows the comparison between the percentages of Htau and amyloid-β phases in brain versus olfactory bulb hTau NTs, NFTs, alpha-synuclein, Aβ in OB, vascular amyloid and intraneuronal amyloid by decades 1-2 and 3-4. The earliest finding in children are hTau NTs follow by LNs and NFTs later. Amyloid plaques, mostly diffuse and few, are stationary throughout the four decades, while amyloid neuronal accumulation and α-Syn increased with age. In a model where $CPM_{2\cdot5}$, age and APOE status were included as suicide predictors, having an APOE4 significantly increases the odds of dying by suicide

4.57 times (p = 0.0025), and 13.1, 10.3, 9.8, 9.4 and 6.3 times higher odds of OB vascular amyloid, neuronal amyloid accumulation, α Syn, hTau NFTs and NTs (all p < 0.0001) respectively, v APOE4 non-carriers having similar $CPM_{2\text{-}5}$ exposure and age.

Neuropathology

The hTau and β amyloid brains' scoring was done in a previous paper. [20] Both the olfactory bulb scoring for hTau NTs and NFTs, β amyloid, and α-synuclein and the brain scoring are seen in Table 1 Suppl

First decade findings

The architecture of the OB layers in the 6 children ≤7 y, was largely preserved. However, there were significant variations in the definition of the layers, particularly the mitral/tufted and the glomerular layers (Figures 3 A, B). In addition, the size and compactness of the glomeruli varied significantly among children (Figures 3 B, C, D, H). All six children were APOE 3/3 and all were classified in a previous work as pretangle stages a-c, 1a, 1b.[20] hTau threads were seen in 5/6 children ages 11 months to 7 years, and a 3y old child had also vascular amyloid, Aβ42 immunoreactivity (IR) in neurons and glial cells around glomerular structures and diffuse amyloid positive areas (Figures 3 D, H). He also exhibited positive hTau and α-Syn IR (Figures 3 E, F, G). A two year old displayed isolated cells packed with particulate matter (Figure 3 B Insert).

Second decade findings

Disorganization of the OB layers and small, irregular and loose glomeruli, some with areas of calcification were striking findings (Figures 4 A, B, C, G, H, J,M). The mitral cells were

difficult to define in relation to granular cell layers (Figures 4 A, H, I, M). Severe disruption of the granule cell layer was present in teens (Figure 4 M). The vascular changes become striking, with prominent endothelial cells and thick walls (Figure 4 A-C). We had 6 subjects ≤ 20 years, with nuclear hTau involving the glomerular layers and the anterior olfactory nucleus (Figures 4 N, O, P), but very few hTau neurites in the AON (Figure 4 INSERT P) or elsewhere. Eight-six percent had hTau mostly as threads or as small tangles (50/57), while 77.2% have vascular amyloid (44/57), and 60% (34/57) had mild diffuse amyloid plaques. Interestingly, we had 5 teens with no IR to hTau that had positive β amyloid either in blood vessels or neuronal. Lewy neurites and/or α-Syn aggregates in the somatodendritic compartment were seen in 76% (43/57) of cases. It is important to note, the anterior olfactory nucleus (AON) was rarely involved in the deposition of abnormal proteins other that increased IR to neuronal amyloid and/or nuclear hTau (Figure 4 P, Q). However, we saw subjects with significant obliteration of the AON by corporae amylacea (Figure 4 R). In this age group, we did not see amyloid plaques or α-Synuclein in the AON, nor we saw Lewy bodies anywhere. A clear example of the severity of the aggregated abnormal protein deposition was an 11 y old boy APOE3 (#7 in Table 1 Suppl), resident in a SW borough, showing extensive β- amyloid and alpha synuclein (Figure 4 D, E,S).The same child had accumulation of particulate matter in glomeruli neurons (Figures 4 G).

Third and fourth decade findings

Striking findings included extensive deposition of corporae amylacea (CA) in subjects carrying an APOE 4 allele (Figures 5 A, B, C, E). Disorganization of the OB layers with few irregular and small glomeruli were striking findings (Figure 5 D).The anterior olfactory nucleus in many cases was almost completely substituted by massive amounts of CA

(Figure 5 E). hTau neurites and NFTs can be seen in glomerular and granular cell layers and white matter tracts (Figures 5 F, G, H). Alpha-synuclein is seen as Lewy neurites and aggregates in the somatodendritic compartment (Figure 5 I, J), but few distinguishable core-and-halo appearance Lewy bodies. Amyloid plaques, intracytoplasmic neuronal accumulation, and vascular amyloid pathology were frequent findings (Figure 5 K).

One micron Toluidine blue sections and Electron Microscopy

One micron toluidine blue and electron microscopy findings were striking in relation to damage to unmyelinated and myelinated axons and blood vessels with abnormal basement membranes and hyperplastic smooth muscle cells in young teens (Figure 6 A-D). Extensive accumulation of lipofuscin distinguished APOE 4 from APOE3 carriers (Figure 6 C, D,E). The endothelial cell (EC) erythrophagocytosis was particularly prominent in APOE 4 children (Figure 6 G, H, I). Clusters of nanoparticles were common between red blood cells in capillaries (Figure 6 J, K). Children and teens also show significant accumulation of lipofuscin (Lf) in endothelial cells, pericytes, smooth muscle cells, and neurons. Abnormal neurovascular units were noted, and isolated beta pleaded sheet helicoidal conformation fibers (Figure 6 F) were observed along with CDNPs of sizes ranging from 9-60 nm. Extensive loss of unmyelinated and myelinated axons is seen in teens and young adults (Figure 7 A-C).The few surviving myelinated axons exhibit numerous CDNPs in their myelin sheets (Figure 7A). CDNPs are seen within neurons and glial cells in target organelles including mitochondria, endoplasmic reticulum (ER), mitochondria-ER contacts (MERC) as well as in nuclear chromatin (Figures 7 B-J). CDNPs are present in damaged dendrites (Figures 7 H, I). The measurable size of the CDNPs were in the 9-60 nm range.

Discussion

Damage to the olfactory bulb (OB) in young Metropolitan Mexico City residents is early, progressive, exhibits Alzheimer and alpha-synucleinopathies hallmarks, and the damage is particularly severe in APOE 4 carriers. The neuropathology in children and teens strongly suggests the OB is an unavoidable target of pollution and nanoparticles likely play a critical role. The neurovascular unit [48] is an early, critical target and active endothelial phagocytosis of RBC fragments containing combustion-derived nanoparticles (CDNPs) is an ongoing phenomenon. The significant vascular and extensive damage to unmyelinated and myelinated axons in the olfactory tracts and the hallmarks of the most prevalent tauopathies and synucleinopathies, obligates us to consider the olfactory bulb as a *sentinel* for evolving neurodegenerative processes. Strong support for pediatric and young adults olfactory testing is an expected outcome of this work.

The OB presence of β-amyloid, abnormal tau and α-synuclein pathology have been described by our classic neuropathologists. [32-47] The strong association of neurodegenerative OB pathology with olfaction deficits and targeted neuronal groups are also well known.[49-54] A key concept in this discussion was put forward by Spires-Jones et al., [55] "*Neurodegenerative diseases such as Alzheimer's disease, Lewy body disease (LBD), Parkinson's disease (PD) …have in common that protein aggregates represent pathological hallmark lesions"*. We are indeed describing an overlap of hallmarks for AD, PD, LBD, etc., in megacity residents, suggesting that in the setting of air pollution we ought to have common etiopathology denominators. Alzheimer's pathology in the olfactory bulb is present in the majority of patients with neuropathologically confirmed AD.[38] In Attems et al., study of 536 autopsies (232 controls) with a mean age of 81.3±0.46 years,

33.8% had a confirmed AD diagnosis. The AD cases showed OB hTau in 98. 3%, 51.7% had Aβ and 34.4% α-Syn pathology. [38] In controls (n: 232), hTau pathology was present in 47.1%, α-Syn in 28.6% and Aβ pathology in 3.5%. Clinically demented cases in Attems et al., work showed significantly higher OB hTau, Aβ and α-Syn scores than non-demented cases.[38] In Tsuboi et al., work, anterior olfactory nucleus (AON) hTau pathology was absent in the lower Braak stages and progressively increased to a 100% involvement in stages V and VI. [34] hTau AON pathology was very rare in their 15 controls with no significant neurodegenerative pathology.[34] In their LBD cases, 77% had α-Syn in the AON and α-Syn AON pathology was only rarely detected in the absence of concomitant hTau pathology. [34] Tsuboi et al., also discussed that APOE4 correlated with the severity of tau pathology in the AON in a gene dose-dependent manner. [34]

In sharp contrast to the OB pathology described in advanced Alzheimer's, LBD and Parkinson's disease elderly patients [32-34, 36-47,56], MMC young residents exhibited hTau in the axon initial segment (AIS) and in neurites as the very first OB tract findings, followed by α-Syn neurites in the glomerular and granular cell regions and the OB tracts. A striking finding –previously described in MMC exposed toddlers and teens in brainstem and supratentorial neurons, ependymal and endothelial cells[20]- was the presence of nuclear hTau in neuronal glomerular and granular layers and the AON in teens. The nuclear hTau would fail to efficiently protect DNA from oxidative stress as commented by Sultan et al., it will *contribute to functional failure of neurons early in life*.[57] The Aβ pathology in the form of diffuse plaques was mostly mild and remained very stable throughout the first 4 decades of life.

It is important to emphasize, the AON was not a target of Htau and/or α-Syn in the first 4 decades, strikingly however, the increase in AON corporae amylacea was significant, particularly in APOE4 carriers. A key finding was the accelerated OB pathology course relative to Braak early subcortical stages a-c, cortical lesions stages 1a, 1b, I and II and NFT stages[45], thus in the first two decades when the majority of children and teens exhibit pretangle stages 1a and 1b, 84% have already OB hTau neurites (Figure 2), strongly suggesting olfactory deficits could be an early guide of early AD hTau stages.

α-Syn is a different story, we have described LNs in brainstem and ENS in children, [12, 13] coinciding with the early OB α-Syn neurites in 68% of subjects in the first two decades. MMC children would be at Lewy pathology stages 1 and 2 according with the distribution of Lewy pathology in sporadic Parkinson's disease in Del Tredeci and Braak work. [41] This PD staging is critical because we are recording autonomic symptomatology in > 60% of the young adult MMC population ages 20.5±1.08 (Personal communication: Nora Vacaseydel-Aceves and Samuel C. Luévano-Castro, April 9, 2018).

The α-Syn OB location is important given the work of Ubeda-Bañon, Saiz-Sánchez and Markram et al., groups. [50-52, 58] Axons of sensory olfactory cells make synapsis with apical dendrites of mitral and tufted cells-the principal cells in the OB- in the glomeruli. Interneurons constitute 20-30% of the neuronal OB population, mostly granule or periglomerular cells. [58] These regions are precisely the location of the first LB neurites we observed in MMC youngsters, while the tertiary structures are not yet involved. Tertiary olfactory-recipient structures [59] including the AON in their bulbar, intrapeduncular and retrobulbar portions are significantly involved in PD, LBD and AD [35,38,52, 60-63]

A key element of vasopathology in the OB is the endothelial engagement in erythrophagocytosis.[64] The circulating RBC are innate carriers tolerating millions of nanoparticles under experimental conditions, and having biocompatibility, low immunogenicity, flexibility, and long systemic circulation. [65-67] RBC are exposed to oxidative stress related to iron containing magnetite nanoparticles [3, 5, 20, 68, 69] with the detrimental combination of high redox activity, surface charge, and strongly magnetic behavior. Experimentally, RBC carrying NPs can get in close proximity to the endothelial surface and binding takes place, this is very important in highly exposed air pollution residents because endothelium adhesion efficiency of RBC increases with their enhanced phosphatidylserine exposure. [70-72] To complicate matters, the phosphatidylserine exposure by RBC is a powerful signal that initiates their phagocytic removal from circulation, a process that normally takes place in liver and spleen. Fens et al., [64] discussed a very similar scenario when they exposed their RBC to oxidative stress, erythrophagocitosis was a common event with the resultant cytotoxicity. Fens et al., suggested and we fully agreed "*significant erythrophagocitosis can induce endothelial cell loss….*"[64] and a major damage to the OBs neurovascular unit. Why is this issue very relevant to city dwellers? Because odor stimulation induces capillary vascular responses that according to Chaigneau et al., are odorant and glomerulus-specific in rats. [73] Thus, since the responses will either increase or decrease RBC flow and in turn proper capillary vascular responses relate to synaptic activation, abnormal RBC and sticky endothelium will have detrimental glomeruli synaptic activation.

Since nanoparticles are ubiquitous in OBs, factors related to access and transportation of NPs and aggregation and propagation of abnormal proteins in the OB and elsewhere in the

CNS and ENS are important. [74-81] Size, shape, surface charge and chemistry, chemical and biocorona composition, and solubility of NPs will be key in their degree of cytotoxicity and genotoxicity, their capacity to cause damage to target organelles and to produce aggregation and propagation of abnormal proteins in nervous tissues. Recently described nanocluster aerosol particles emitted by road transportation (1.3-3.0 nm) potentially acting as nanosized condensation nuclei for the condensation of atmospheric low-volatile organic compounds, ought to be of great concern for OBs damage.[75] Sintov et al.,[76] summarize three pathways involved in the transport of NPs through the OB i. axonal transport, ii. transcellular transport across the supporting cells in the olfactory region, iii. paracellular diffusion between supporting and neural cells. In the work of Wang et al., [79] 35-50 nm Fe2O3 NPs instilled intranasally, were readily located by TEM in the axons of olfactory neurons and in mitochondria and lysosomes of hippocampus cells in exposed mice. In the work of Alvarez et al.,[74] gold NPs produced a strong acceleration of α-synuclein aggregation, the effects were dependent on the NPs size and concentration, being strongest for NPs 10 nm in diameter, which produced a 3-fold increase in the overall aggregation rate at concentrations as low as 20 nM. Since the NPs identified in Mexico City residents are iron highly magnetic NPs [5] the work of Xie et al., [80] describing oxidative stress, lipid peroxidation and depletion of superoxide dismutase (SOD), glutathione, and catalase (CAT) activities upon iron oxide NPs exposures is very relevant to OBs damage. An additional factor of great interest would be *magnetic fields*. Min et al., [81] argue that the rate of iron NP uptake and transport across cell monolayers is enhanced by a pulsed magnetic field and significantly inhibited at low temperature under both constant and pulsed magnetic field conditions, consistent with an active mechanism such as endocytosis, mediating NP transport. Thus, *environmental exposures to pulsed magnetic fields* could be

another factor in the equation to compare *transport and damage* of iron oxide NPs across populations.

We have described different sizes of NPs in different neural regions both in humans and dogs, *so size matters*.[3,13,20,68] In the current OB study measurable NPs were in the range of 9-60 nm, thus included sizes ~10nm, very efficient in α-synuclein aggregation.[74]

The relationship between APOE4 status, suicide risk, depression, olfaction deficits, and cumulative $PM_{2.5}$ deserves extensive research.[20] We found APOE4 carriers have 4.57 times higher suicide odds, and higher odds of OB AD and α Syn pathology. These findings are critical for several reasons: i. We fully expect a relationship between the OB neuropathology and olfactory deficits, a relationship that is clear in older populations with both AD and alpha-synucleinopathies.[33,34,36,38,53,63] ii. There are a significant number of papers on depression, OB size, and emotions.[82-86] The key question is how many young subjects with olfaction deficits, depression and altered emotional responses are already in their way to AD or PD/DLB?

Finally, there is an issue we are obligated to discuss: what is the ultimate importance of the OB neuropathology spectrum in young highly exposed individuals and without any significant extraneural pathology? There is clear evidence of neurovascular unit damage with increased lipofuscin (Lf) as a striking finding. Lf formation is driven by ROS, is an intralysosomal, non-degradable, auto-fluorescent macromolecule which under physiological circumstances accumulates with age and can affect autophagy - the lysosomal degradation of a cell's constituents.[87] McElnea et al., [87] make a statement that is applicable

to the OB in exposed air pollution populations: *intracellular lipofuscin accumulation may have important effects on autophagy*. Indeed, Lf relates to the rate of oxidative damage to proteins, the functionality of mitochondrial repair systems, the impairment of proteasomal systems, and the functionality and effectiveness of the lysosomes. [88, 89] The issue of the autophagy-lysosome pathway (ALP) regulating intracellular homeostasis of the cytosolic protein SNCA/alpha-synuclein has been discussed by Minakaki et al., [90] Inhibition of the ALP increases fused multivesicular body-autophagosome compartments and the "*autophagosome-exosome-like*" profile and alters the intracellular homeostasis of the cytosolic protein SNCA/alpha-synuclein. Why is Minakaki et al., outstanding work relevant to us? Because is precisely this autophagy-lysosome pathway that is impaired in alpha-synucleinopathies, including PD and DLB.[90] Moreover, iron promotes α-Syn aggregation and transmission. [91] Xiao et al., results are very relevant to our findings (Fe and assorted metals-containing nanoparticles) demonstrated that iron promoted α-synuclein aggregation and transmission by *inhibiting autophagosome-lysosome fusion*. Further, Fe decreased the expression of nuclear transcription factor EB, a transcriptional regulator of autophagosome-lysosome fusion, and inhibited its nuclear translocation through activating AKT/mTORC1 signaling.[91] Thus, we have a distinct plausible pathway for α-synuclein aggregation and transmission.[91]

Corporae amylacea (CA) -glycoprotein-based deposition- in significant numbers were also an outstanding OB finding.[92] In the work of Pirici et al., the three-dimensional structure of CA is complex with branching exhibiting a direct correlation with the diameter of vessels, while perivascular CAs are enclosed in pockets of the basement membranes. Interestingly, endogenous astrocytic heme oxygenase-1 (HO-1)-a cytoprotective enzyme-, reported to be

localized in mitochondria under stress and contributing to preserve mitochondrial function, promotes transformation of normal mitochondria to CA-like inclusions.[93] Thus, several potential CA production pathways could be at work in OBs exposed to hostile conditions. Since the study subjects are young, age certainly is not a factor, in consequence neurodegenerative conditions have to be at play.[94, 95]

There is no question we are seen AD and PD/DLB olfactory bulb hallmarks, along with increased lipofuscin and corporae amylacea. This overlap is what we see as neuropathologists in a demented patient or in a presumably cognitively intact elderly *control*. Braak and Del Tredeci commented about the spectrum of Lewy Body diseases and the fact cognitive impairment precede dementia in sporadic PD patients [42] Also there is plenty of literature about the overlap between vascular disease and tautopathies and alpha synucleinopathies and the impact of vascular risk factors upon PD dementia and dementia with Lewy Bodies versus AD.[96-99] Love and Miners[100] commented on a major contributor to the progressive hypoperfusion seen in AD: endothelin-1 (ET-1)- a marker of endothelial damage significantly increased in Mexico City children.[101-102] ET-1 levels in MC children are positively strongly correlated with daily outdoor hours, and 7-day cumulative levels of PM air pollution < 2.5 um. [102] Thus within the context of ET-1 vasoconstriction and the neurovascular unit damage, both increased cerebrovascular resistance and loss of neurally-mediated vasoreactivity could also play a role in the hypoperfusion effects.[103-104]

Our findings have several limitations: there is an overrepresentation of males, thus we are unable to discuss how the OB pathology progresses in females. Since we had no means of assessing olfactory and neurological data, the direct association with OBs pathology is not possible. This lack may have led to relevant olfactory, psychiatric, behavioral, and

neurotoxic exposure information. On the other hand, based on our clinical studies we know about the olfaction deficits, their relationship with metabolic brain changes, the extensive cognitive deficits, their systemic inflammation, endothelial dysfunction, etc., in a comparable *healthy* population .[10,31, 54,101,102]

We strongly support a complex overlap of tautopathies and alpha-synucleinopathies evolving from childhood with a common denominator: combustion-derived nanoparticles potentially including atmospheric nanocluster aerosols. [5, 75] Since the olfactory bulb is an early target and hTau is a prime actor, olfactory testing should be done along with early cognitive and behavioral testing to identify subjects at high neurodegenerative risk. APOE 4 carriers should start neuroprotective interventions in the first two decades of life.

A key challenge is to define clinical, laboratory, imaging, and cognitive *non-invasive* markers for the initial stages of the evolving complex tautopathies and alpha-synucleinopathies. It is imperative that we understand the earliest neuropathological changes upon exposures to air pollutants, the complexity of the interaction between sources and characteristics of pollutants and the ultimate CNS manifestations which will vary with age, nutritional, metabolic and genetic interactions.

Early interventions should be integrated in health and educational agendas along with identifying early gender-specific risk trajectories. We are certain air pollution should be included as an early risk factor in the research priorities to reduce global burden of dementia, ignoring the subject is not in the best interest of millions of exposed people. Pollution control should be prioritized, and supporting research related to air pollution and pediatric, teens and young adults neurodegenerative impact ought to be a goal in our prevention efforts to stop these diseases. Screening for olfaction deficits early in life,

certainly in the first 2 decades of life would help to define cohorts at highest risk and provide mechanistic insights into major neurodegenerative fatal diseases including Alzheimer and Parkinson's. Preventive medicine ought to be our goal and we must consider the ramifications of lifelong air pollutant exposures on children and do what we can to protect them.

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105.

FIGURES April 24, 2018

Figure 1

Cumulated $PM_{2.5}$ trends of the annual, averaged over 3 years, mean concentrations in excess the USAEPA standard for 179 individuals according to their age at the time of death and residential location. The regressions are overlapped on a map showing the spatial distribution of the annual $PM_{2.5}$ concentrations for the base year 2008 (the last year of the 5 year study). The map in the upper right corner shows the spatial distribution of the annual average of the daily ozone 8-h maximum for 2008.

Figure 2

Percentages of hTau stages as defined by Braak et al., [43-45] in subcortical and cortical stages in previous study.[20] Data compared to olfactory bulb percentages of hTau NTs (neurites) and NFTs (neurofibrillary tangles), and α-synuclein (OB α-Syn) [38-40] in the same subjects by age: 0-20 and 21-40 years at the time of death.

Brain Htau Stage [43-45]: 0=absent, 1= pretangle stages a-c, 2= pretangle stages 1a, 1b, 3=NFT stages I, II, 4=NFT stages III-IV, 5=NFT stages V-VI

Olfactory bulb: Htau scores NTs and NFTs separately: 0=absent, 0.5=very mild, only singly lesions, 1=mild, 2=moderate, 3=severe.[38, 39]

α-Synuclein scores: 0=absent, 1: mild, sparce Lewy neurites or Lewy bodies, 2:moderate, more than 1 Lewy body in a low power field (LPF) and sparce LNs, 3:severe >1 LBs and scattered LNs in a LPF, 4: very severe, numerous LNs and LBs according to the templates published by the Dementia with Lewy Bodies Consortium. [40]

Figure 3

Representative immunohistochemistry (IHC) and H&E sections from children in the first decade of life.

A. Two year old male APOE 3/3, olfactory bulb 7 μm thick section. The anatomical organization of the olfactory bulb is still intact and the different layers could be defined: GL glomerular layer, ML mitral layer and GRANL granular cell layer. The olfactory tract (OT) is unremarkable. H&E, Scale bar 200 μm

B. Same child as A. Higher power shows focal disorganization of the OB architecture with isolated mitral neurons (arrow) H&E, Scale bar 100 μm. INSERT: Olfactory tract isolated cells showed abundant particulate material. H&E, Scale bar 10μm

C. Three year old APOE 3/3. Glomeruli are abundant, with significant variation in size. Abnormal blood vessels with no visible lumen are seen throughout the sample (arrow). H&E, Scale bar 50μm

D. Same child as C. Glomerular layer showed numerous immunoreactive (IR) amyloid β cells (brown product). IHC x Aβ counterstained with H, Scale bar 100μm.INSERT: Higher power to show glomerular and periglomerular cells with cytoplasmic IR to Aβ. Scale bar 10 μm

E. Glomerular (g) region shows isolated hTau IR in axon initial segment (AIS) (arrow), contrasting with the negative background. IHC x AT8 without counterstain, Scale bar 40μm

F. Granular cell layer, same child as E showing one hTau positive aggregated IR in axon initial segment (AIS). IHC xAT8 Scale bar 40µm

G. Same child as C with α-Synuclein IR in glomerular region. IHC x α-Syn phosphorylated at Ser-129, LB509, Scale bar 10µm

H. An isolated glomerular immunoreactive (IR) area to amyloid β. ICH x Aβ, Scale bar 50 µm

Figure 4

Representative immunohistochemistry (IHC) and H&E sections from children and young adults in the second decade of life.

A. Eleven year old boy APOE 3/3. The laminar organization of the olfactory bulb is still visible (glomerular layer, GL), but the different layers are ill-defined. There is significant variation in the glomeruli size (g) and blood vessels are prominently seen (arrows).H&E, Scale bar 100 µm
B. A close-up of the glomerular region (g) to show the presence of abnormal blood vessels with significant reduction in their lumen (arrowheads). H&E, Scale bar 50 µm
C. Close-up of an abnormal blood vessel in glomerular region. Notice a polymorphonuclear leucocyte (arrow) attached to the vessel wall, and the vacuolated endothelial cells (arrowhead). H&E Scale bar 50 µm
D. Diffuse amyloid plaques are seen throughout the OB layers (arrow). IHC x Aβ counterstained with H, Scale bar 100µm.

E. Same child as C with extensive Aβ IR deposits of different sizes in the olfactory tract (arrow) IHC x Aβ counterstained with H, Scale bar 100µm.INSERT: Upper left, discrete isolated diffuse Aβ plaques and upper right Aβ IR in larger arterial vessels in the arachnoidal space.

F. Eleven year old boy (same as A) with extensive α-Synuclein IR in glomerular region. IHC x α-Syn, LB509, red product, Scale bar 50µm

G. The glomerular region is a target for accumulation of particulate material. Several cells within the glomerulus and outside are packed with particles (arrows). H&E, Scale bar 10 µm

H. Fourteen year old boy APOE 3/3 with striking variation in glomeruli size, some (g) are basically amorphous, without visible nuclei and very small and irregular. H&E, Scale bar 50 µm. INSERT: This child had moderate hTau neurites. IHC xAT8 Scale bar 10µm

I. Seventeen year old boy APOE 3/3 with a significant abnormal laminar organization of the olfactory bulb and poor definition of the different layers (granular layer,left). The glomeruli region (right side of the picture) shows very amorphous and pale glomeruli. H&E, Scale bar 10 µm

J. Same 17y old boy as in I to show a glomerulus with an area of metaplastic calcification (framed in a square). H&E, Scale bar 20 µm

K. Same teen as J,I. Extensive IR to α-synuclein. Neuronal perikaryal inclusions (arrows) and Lewy neurites are present. IHC x α-Syn, LB509, red product, Scale bar 50µm

L. Twenty year old young man APOE 3/4. Numerous amyloid β diffuse plaques scattered throughout the specimen (arrows). IHC x Aβ counterstained with H, Scale bar 200µm

M. Same subject as L. Severe disorganization of the laminar normal architecture with the glomerular region showing few and amorphous, small glomeruli (g), few clusters of mitral neurons (arrows) and a thin granular cell layer (GRANL). H&E, Scale bar 50µm

N. Glomerular layer in the 20 y old APOE 3/4 subject. Nuclear Htau was significant in this subject and few small Htau plaques (head arrow). IHC xAT8 Scale bar 50µm

O. Same subject as N to show the glomerular region stained for Htau and counterstained with H. There is strong nuclear Htau IR and cytoplasmic, granular Htau in the perikaryon of a periglomerular cell (headarrow).Notice the longitudinal segment of a blood vessel (BV) in close proximity to the central glomerulus. We marked the enlarged Virchow-Robin space with (*).

P. The anterior olfactory nucleus in this 20y old male APOE 3/4 shows extensive nuclear Htau(arrows) but no IR with either NTs or NFTs. IHC xAT8 Scale bar 50µm

Q. Abeta in AON

R. The same young man shows extensive IR to α-synuclein in the glomerular layer. Neuronal perikaryal inclusions (arrows) are abundant. IHC x α-Syn, LB509, red product, Scale bar 10µm

Figure 5

Representative immunohistochemistry and H&E sections from subjects in the third and fourth decades of life.

A. Thirty two year old female, APOE 4/4. Olfactory tracts of APOE4 carriers were characterized by extensive deposition of corporae amylacea (CA) and severe rarefaction of white matter tracts. H & E, Scale bar 100 μm
B. Same subject as A olfactory bulb in the region of the anterior olfactory nucleus (AON), massively occupied by corporae amylacea. H & E, Scale bar 100 μm
C. Higher power of the AON area to show the corporae amylacea deposition (H&E Scale bar 50 μm) and the presence of astrocytes with hyperchromatic, convoluted nuclei (arrowhead) containing CA in their cytoplasm. INSERT: abnormal astrocyte with large CA, note the abnormal surrounding neuropil. H&E Scale bar 10 μm.
D. APOE 4 carrier, the glomeruli (g) are very small and amorphous. A few mitral neurons remain (arrows). H&E Scale bar 50 μm
E. Twenty-five year old male, APOE 3/4, suicide, anterior olfactory nucleus AON completely occupied by corporae amylacea CA. H & E, Scale bar 50 μm
F. Forty year old male, APOE 3/3 moderate hTau NTs (arrowheads) and NFTs(arrows) in glomerular layer IHC xAT8 Scale bar 20μm
G. Thirty nine year old male, hTau NTs (arrows) and NFT(arrowhead) in granular layer IHC xAT8 Scale bar 10μm
H. Same subject as G to show hTau NFTs and NTs in granular cell layer. IHC xAT8 Scale bar 10μm

I. Twenty-eight year old female, APOE 3/4 (#106 in the Table 1 Suppl). Extensive IR α-synuclein LNs. IHC x α-Syn, LB509, brown product, Scale bar 50µm. INSERT LEFT: Neuronal perikaryal inclusions are also seen. INSERT RIGHT: Enlarged IR neurites are common. Both inserts: IHC x α-Syn, LB509, red product, Scale bar 10µm

J. Twenty-seven year old male, APOE 3/4 (#97 in the Table 1 Suppl). Neuronal perikaryal α-synuclein IR (arrows) and neurites(arrowheads). IHC x α-Syn, LB509, brown product, Scale bar 10µm

K. Twenty-seven year old male, APOE 3/3 with a CPM2.5 of 2303 µg/m3 has no identifiable AON neurons, a few cells have IR to amyloid β (arrows) and an isolated diffuse amyloid plaque (arrowhead). IHC x Aβ counterstained with H, Scale bar 100µm. INSERT: Aβ IR arteriole. Scale bar 10µm

Figure 6

Representative 1µm toluidine blue and electron micrographs pictures.

A. Fourteen year old girl APOE 3/3. Blood vessel basement membranes are focally thick (arrowhead), mild enlargement of the Virchow-Robin space (*) is noted and there is a significant loss of both myelinated(arrows) and unmyelinated axons. A few myelinated axons remain in the olfactory tract (arrows). Toluidine blue, Scale bar 10µm

B. Same 14 year old as A, section of mitral tufted cell layer. Few small myelinated axons remain and unmyelinated axons are difficult to identify. Small clusters of

thin myelinated axons marked by short arrows and mitral neurons by longer arrows.. Toluidine blue, Scale bar 10μm

C. Olfactory bulb in a 17 year old male APOE 3/3. Numerous Lipofuscin (Lf) granules are seen. Red blood cells in the lumen of the vessels are marked RBC. Scale bar 2 μm

D. A close-up of a cluster of Lipofuscin granules in the cytoplasm of an endothelial cell. Scale bar 200 nm.

E. Endothelial cells exhibit extensive deposit of lipofuscin and RBC are in close contact with endothelial cells (EC). Scale bar 500 nm.

F. Fourth teen year old girl with beta pleaded sheet helicoidal conformation fibers in the cytoplasm of an endothelial cell (EC) (lower half of the picture). Numerous CDNPs are seen in the EC nucleus and in the EC cytoplasm (arrows). Lf marks lipofuscin in close contact with the nucleus of the endothelial cells. Scale bar 500 nm

G. Seventeen year old male APOE 3/4. The endothelial cells of small blood vessels are involved in active erytrophagocitosis (square frame). Scale bar 2 μm

H. A close-up shows the square frame from G: one red blood cell (RBC) is surrounded by a membranous lysosomal structure in an endothelial cell (EC). The nucleus(N) of the EC is closed to the lysosomal structure. Scale bar 500 nm

I. Same 17y old subject as C. The endothelial cell is phagocytizing a cellular non-identified fragment also containing numerous NPs (arrowhead). NPs are marked with a long arrows. The * marks apparently empty EC vacuoles. RBC are seen in the lumen of the vessel. Scale bar 500 nm

J. Capillaries are commonly occupied with red blood cells (RBC) containing significant amounts of NPs that orient themselves in a line between them (arrowhead). Scale bar 500 nm

K. A capillary in a 15 year old male. The NPs are also seen mostly between 2 RBC (arrowheads). Interestingly, a lipofuscin (Lf) early granule in the endothelial cytoplasm (EC) shows a rim of NPs (black arrowhead). Scale bar 500 nm

Figure 7

Representative 1μm toluidine blue and electron micrographs pictures.

A. Fourteen year old girl APOE 3/3 medium size blood vessel in lower portion of picture. Several myelinated axons of different caliber, all show focal fragmentation of myelin and clusters of particles (arrowheads). Empty spaces in the neuropil are marked (*) and one isolated combustion-derived nanoparticle is marked (arrow). Scale bar 2 μm

B. Twenty-four year old male APOE 3/3. Capillaries with hyperplastic endothelial cells (*) are surrounded by a glial cell (arrow heads). Scale bar 2 μm

C. Same subject, an oligodendrocyte (arrow) is surrounded by a few abnormal myelinated axons, some with very thin myelin (arrowhead, lower right). Scale bar 2 μm

D. A close-up of the oligodendrocyte to show abnormal nuclear membrane pores and NPs inside the nucleus (upper arrow). NPs are also present in the cytoplasm (lower left arrow) and mitochondria (M) are abnormal. Lipofuscin granules are also present in the cytoplasm (Lf). Scale bar 500 nm

E. A higher power of D to show the relationship between the dilated endoplasmic reticulum (ER) and the lysosomal Lf structure. The short arrow points to the space between the ER and the Lf. The larger arrow points to the proximity between the mitochondria and the ER. Scale bar 500 nm

F. Twenty year old male APOE 3/3. Mitochondria in unmyelinated axons contain combustion-derived nanoparticles (right arrow). Elsewhere, NPs are seen in severely damaged unmyelinated axons (left arrow). Empty dendrite-like structures are marked (*). Scale bar 500 nm

G. A common finding in between unmyelinated axons was the presence of clusters of nanoparticles (arrows). Empty structures are common (*).Scale bar 500 nm

H. A severely abnormal dendrite with a combustion-derived nanoparticle (arrowhead). Scale bar nm

I. Nanoparticles are seen inside dendrites and unmyelinated axons (arrows). Scale bar nm

J. Empty structures and abnormal mitochondria with combustion-derived particles are ubiquitous. Scale bar nm