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## Abstract

Severe air pollution exposures produce systemic, respiratory, myocardial, and brain inflammation and Alzheimer's disease (AD) hallmarks in clinically healthy children. We tested whether hippocampal metabolite ratios are associated with contrasting levels of air pollution, APOE and BMI in paired healthy children and one parent sharing the same APOE alleles. We used (1) H-MRS to interrogate bilateral hippocampal single-voxel in 57 children ( $12.45 \pm 3.4$  years) and their 48 parents ( $37.5 \pm 6.78$  years) low pollution city v Mexico City (MC). NAA/Cr, Cho/Cr, and mI/Cr metabolite ratios were analysed. The right hippocampus N-acetylaspartate/creatine (NAA/Cr) was significantly different between cohorts ( $p=0.007$ ). The NAA/Cr ratio in right hippocampus: controls v APOE 4 MC children and left hippocampus: MC APOE 4 parents v their children was significantly different after adjusting for age, gender and BMI ( $p=0.027$  and  $0.01$ , respectively). The NAA/Cr ratio is considered reflective of neuronal density/functional integrity /loss of synapses/higher pTau burden, thus a significant decrease in hippocampal NAA/Cr ratios may constitute a spectral marker of early neurodegeneration in young urbanites. Decreases in NAA/Cr correlate well with cognitive function, behavioural symptoms and dementia severity, thus since the progression of AD starts decades before clinical diagnosis, our findings support the hypothesis that under chronic exposures to fine particulate matter and ozone above the standards, neurodegenerative processes start in childhood and APOE 4 carriers are at higher risk. Gene /environmental factors are critical in the development of Alzheimer's disease and the identification and neuroprotection of young urbanites at high risk must become a public health priority.

**Keywords:** APOE, air pollution, BMI, children, memory, MRS, hippocampus, NAA/Cr ratio, neuroprotection, PM<sub>2.5</sub>

## 1. Introduction

There is significant concern about the effects of air pollutants upon the developing brain [1-16]. We previously published that in a young cohort of  $18.37 \pm 6.9$  year old Mexico City (MC) subjects, apolipoprotein E (APOE) epsilon 4 carriers had greater frontal hyperphosphorylated tau and diffuse A $\beta$  plaques versus E3 carriers ( $Q = 7.82$ ,  $p = 0.005$ ) [7]. Mexico City APOE  $\epsilon 4$  versus  $\epsilon 3$  children age  $13.4 \pm 4.8$  years had a reduced NAA/Cr ratio in the right frontal white matter and decrements on attention, short-term memory, and below-average scores in Verbal and Full Scale IQ (>10 points) [14]. These observations are critical for young APOE 4 carriers residing in polluted environments and beg the question of the involvement of the hippocampus in the detrimental air pollution responses. We have shown that in APOE 4 and 3 Mexico City v controls aged  $25.1 \pm 1.5$  years upregulation of cyclooxygenase-2, interleukin-1 $\beta$ , and CD14 in olfactory bulb, frontal cortex, substantia nigrae and vagus nerves contrasted with hippocampal cyclooxygenase-2 (COX2) not reaching significance, while IL1 $\beta$  mRNA had a p value of 0.06, suggesting an evolving inflammatory trend [11]. On the other hand and very relevant to our MRS current findings, measurements of mRNA COX2 in the hippocampus of Mexico City residents age  $51.2 \pm 4.9$  years versus controls age  $58.1 \pm 4.6$  years showed a significant difference, marking an established hippocampal inflammatory process with age in cognitively intact urban individuals [17]. Our logical approach was then to study pairs of healthy parents and children from low and high air pollution areas and focus on the hippocampal metabolites using Proton Magnetic Resonance Spectroscopy ((1) H-MRS).

Given the decrements on attention, and short-term memory in Mexico City APOE 4 v 3 children [14], our interest for this work was to determine if APOE plays a critical role in the metabolic hippocampal responses, key for neurobiological processes underlying learning and memory. The apolipoprotein E 4 allele is the strongest known genetic risk factor for Alzheimer's disease and APOE has multiple functions in regulating A $\beta$  clearance and aggregation, impacting also AD pathology independent of A $\beta$  [18, 19]. Brain structure and function are closely associated with APOE genotype not just for AD but also in healthy elderly individuals, as well as in asymptomatic young individuals and infants [20-22]. Also key for this work, published reports have established associations between adiposity and hippocampal-dependent and hippocampal-independent memory among prepubertal children [23], interactions of body mass index and apolipoprotein E  $\epsilon 4$  allele on cognitive decline [24], and the controversial issue of middle age high BMI and risk of dementia [25], thus we include BMI as a key variable.

Air pollution is a major public health issue in Mexico City and since children and young adults are chronically exposed to high concentrations of fine particulate matter and ozone and already show the neuropathological hallmarks of both Alzheimer and Parkinson's diseases [7,8,11,15,16], and given the recent literature supporting a strong association between the risk of AD [26] and smaller total cerebral brain volume in older adults [27] with exposure to elevated levels of PM<sub>2.5</sub>, the paediatric urban high AD risk issue deserves immediate attention.

Thus, we aimed at identifying possible associations in clinically healthy children and their parents, residents in Mexico City v a clean city between APOE genotype, BMI and the emergence of hippocampal metabolic alterations. Achieving these aims may lead to 1. A definition of the temporality of the metabolic involvement of the hippocampus in child/parent pairs in the setting of severe lifetime air pollution exposures, 2. The role of APOE in hippocampal metabolic responses and their interpretation, 3. The impact of the body mass index on hippocampal responses, 4. The implementation of neuroprotective interventions.

Our long term focus is the early identification of AD high risk young individuals and the development of paediatric Alzheimer's disease prevention strategies that could be applicable worldwide.

## 2. Procedure

This prospective protocol was approved by the review boards and ethics committees at involved institutions, written consent was obtained from parents and verbal consent from children.

### 2.1 Study Areas

Mexico City Metropolitan Area is the largest urban centre in North America and is an example of extreme of urban growth and accompanying environmental pollution [28, 29]. The metropolitan area of over 2000 square kilometres lies in an elevated basin 2240 m above sea level surrounded on three sides by mountain ridges, a broad opening to the north and a narrower gap to the south-southwest. Twenty-four million inhabitants, over 50 000 industries and >5 million vehicles consume more than 50 million litres of petroleum fuels per day [29-30]. Ozone concentrations peak towards the downwind southwest area in the afternoon as a result of the typical diurnal wind transport of air polluted masses coming from the urban area. The higher 8-hr O<sub>3</sub> and PM<sub>2.5</sub> concentrations coincide with the times children are outdoors during the school recess and physical education periods and when they play outdoors at home [10].

### 2.2 Participant Children

This work includes data from 57 right-handed children from Mexico City and the control city (*Mean age* = 12.45 years, *SD* = 3.4). For this study, we included 9 control children from Polotitlán (*Mean age* = 9.77 *SD* = 0.83 years). In the MCMA cohort we identified 27 children with an APOE 3/3, and 21 with APOE 4, including two homozygous children (*Mean age* = 13.79 *SD* = 4.68 years). All control children were APOE 3/3. Children's clinical inclusion criteria were: negative smoking history and environmental tobacco/nicotine exposure, lifelong residency in MC or in the control city, residency within 5 miles of the city monitoring stations, full term birth, and unremarkable clinical histories, including absence of history of hospitalizations for respiratory illnesses, ear-nose-throat (ENT) and oral symptomatology and/or surgery, head trauma, systemic or respiratory viral diseases, lower respiratory system illnesses, and personal and family histories of atopic diseases. We specifically excluded children with active participation in team sports with high incidence of head trauma. All included children were taking no medications and had one parent included in the study. 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.

### 2.3 Participant Parents

This work includes data from 48 right-handed parents from Mexico City and the control city (*Mean age* = 37.5 years, *SD* = 6.77). For this study, we included 7 control parents from Polotitlán (*Mean age* = 34.57 *SD* = 6.02 years). In the MC cohort we identified 27 parents with an APOE 3/3, and 14 with APOE 4 sharing the same APOE alleles with their children. All control parents were APOE 3/3. Parents' clinical inclusion criteria were: negative smoking history and environmental tobacco/nicotine exposure, lifelong residency in MC or in the control city, residency within 5 miles of the city monitoring stations and unremarkable clinical histories, including absence of history of hospitalizations for respiratory illnesses, ear-nose-throat (ENT) and oral symptomatology and/or surgery, head trauma, systemic or respiratory viral diseases, lower respiratory system illnesses, hypertension, cardiovascular diseases, diabetes, metabolic syndrome, obesity, and personal and family histories of atopic diseases. We specifically excluded parents with active participation in team sports, head trauma or occupational exposures to toxic substances. Mothers had a negative history of drug intake, including alcohol. All included parents were taking no medications. In the control and MC parent groups, parents had more than one child participating in the study.

### 2.4 Paediatric and Otoneurological Examination

Children had complete clinical histories and physical examinations by our paediatricians. The initial paediatric examination was followed by a complete otolaryngological and neurological examination. All included children were clinically healthy and actively engaged in outdoor activities.

### 2.5 Parents Examination

Parents had complete clinical histories. All included parents were clinically healthy.

## 2.6 Brain Magnetic Spectroscopy Imaging (MRS)

All participants in this study were awake and were not medicated. The scanning technologist was the same for all subjects and blind to all information, except date of birth and gender. MRS scans were acquired on a 1.5 Tesla 5T Signa Excite HD MR (General Electric, Milwaukee WI, USA) with an 8 channel high resolution head coil. Two sets of images were acquired on orthogonal planes: sagittal T1 Fluid Attenuation Inversion Recovery (FLAIR) (TR: 2238 ms; TE: 27.2 ms; TI: 750 ms; NEX: 2; FOV 21x21; 5 mm thickness; 1 mm spacing), and coronal T1 FLAIR (Tr: 1538 ms; TE: 23.7 ms; TI: 708 ms; NEX: 2; FOV 22x17.6; 3.0 mm thickness; 0.0 spacing). <sup>1</sup>H MRS was obtained from a single 8 cm<sup>3</sup> voxel (2 cm x 2 cm x 2 cm), with the Probe-P sequence, TR: 1500 ms; TE 35 ms, with automatic shimming and water suppression, time of scan 2'12". Our voxel selection was done using the criteria of baseline stability, maximum width at half height with a maximum of 10 Hz, and peak definition [31]. There were no gross asymmetries after adequate eddy current correction and the fitting of the baseline followed the phase of the metabolites. Fits deviated from the peak were discarded, and suboptimal voxels were excluded from analysis. Voxel elimination due to artifacts and spectral quality was not different between groups. The center of the hippocampal voxel was placed to bisect the hippocampal anterior-posterior axis, using the T1-coronal images as a guide. One MRS voxel of interest (VOI) for medial hippocampal regions (right and left hemisphere) including the hippocampus and the entorhinal cortex were done for each subject and the same slice was used for both right and left hippocampal voxels (Figure 1). To minimize experimental confounds associated with the placement of MRS VOI, special attention was given to ensure that the same slice covering the same anatomical location and orientation was selected. In addition, all of the subjects were scanned by the same experienced technologist. Metabolite intensities were quantified by referencing to an internal standard creatine (Cr).

## 2.7 Apolipoprotein E Genotyping

APOE genotype information was obtained through analysis of peripheral blood samples for the clinical participants. Samples were genotyped using Taqman ready to use assays from both SNP's that constitute the APOE genotype according to TaqMan Gene Expression Assays, Applied Biosystems, 2006.

## 2.8 Data Analysis

First we calculated the summary statistics, i.e., mean and standard deviations of all variables including age, BMI, hippocampal ratio variables in both children and parents having APOE3 and APOE4 genes in both control and Mexico City cohorts. Moreover, we tested whether each of the hippocampal ratios is significantly different among the cohorts of six combinations of parent-children, APOE genes and residency. We performed Kruskal-Wallis tests and we fitted several linear regressions where each of hippocampal ratio variables was response and age, gender, BMI and residency were predictors. We also performed standard model diagnostics, i.e., tested homoscedasticity and tested for normality of errors. We used Breusch-Pagan test for testing homoscedasticity and Shapiro-Wilk test for testing normality of errors. In the regressions where model assumptions were not satisfied, we either disregarded outlying observation(s) and/or used power transformation of the response. Once we reached reasonable linear models with valid model diagnostics, we tested the statistical significance of residency after adjusting age, gender and BMI. We did this for each pair of Control-MC APOE3, Control-MC APOE 4 and Control-MC and within the gene specific (APOE3 or APOE4) subject pools of children and parents. We also calculated and tested Pearson's partial correlation coefficients of BMI with the hippocampal ratios within each cohort after adjusting age and gender. We performed the statistical analyses using Excel and the statistical software 'R' (<http://www.r-project.org/>).

# 3. Results

## 3.1 Air Pollution Levels

MC children and parents in this study resided in the south and have been exposed to significant concentrations of O<sub>3</sub>, and particulate matter (PM) for their entire life [29]. The climatic conditions in MC are relatively stable thus pollutant concentrations are consistent year after year. For the purposes of this study we analyzed the main criteria pollutants in the period 1997-2012 that include the parents and children residency in MC and the control city. According to data from the government air quality monitoring network [30], during the 2002-2012 period, PM<sub>2.5</sub> annual average concentrations in the representative south monitoring station was 20.49 µg/m<sup>3</sup> (the US EPA annual standard calls for a PM<sub>2.5</sub> annual average below 15 µg/m<sup>3</sup>). In addition, the 4<sup>th</sup> highest daily maximum 8-hr average ozone concentration

in ppm for the same years, in the same area was 0.123 (the ozone US EPA air quality standard stands for an annual fourth highest daily maximum 8h concentration of 0.075ppm). Short term PM<sub>10</sub>, O<sub>3</sub>, SO<sub>2</sub> and NO<sub>2</sub> monitoring campaigns performed by the Government of the State of Mexico in the control city Polotitlán before 2005 [32], recent measurements of PM<sub>10</sub> and O<sub>3</sub> in San Juan del Río, 24 km to the northwest of Polotitlán [33], and mathematical modeling of ozone air quality in the center of Mexico [34] indicate that criteria pollutant control levels have been below the USA EPA air quality standards due to the fortunate combination of few contributing emission sources from industry and cars and good ventilation conditions due to regional winds.

### 3.2 Demographic Data and Physical Exams

Tables 1 and 2 summarize the characteristics of the selected children and parents cohorts. Vital signs and the physical examination results were unremarkable in all participants. There was a significant difference in BMI ( $p=0.02$ ) between APOE 4 v 3 Mexico City children cohorts. BMI was significantly higher in MC parents carrying an APOE 4 allele versus control and Mexico City APOE 3 carriers ( $p=0.04$ ).

### 3.3 MRS results

Table 3 show the MRS hippocampal data for both children and parents. The right hippocampus NAA/Cr and the CHO/Cr ratios were significantly different between the six cohorts using the Kruskal-Wallis test ( $p=0.0074$  and  $0.0406$  respectively). APOE 4 Mexico City children had the lowest NAA/Cr ratios for the right hippocampus (Table 3). There was a significant difference in the right hippocampus NAA/Cr ratio between control children and APOE 4 Mexico City children ( $p=0.027$ ) after adjusting for age, gender and BMI (Table 4). The left hippocampus NAA/Cr ratio was also significantly different between APOE 4 children versus parents ( $p=0.019$ ), the lowest values seen in parents (Table 4). When we analysed correlations between BMI and the different hippocampal ratios, adjusted for age and gender (Table 5), in control parents BMI correlated with right hippocampal NAA/Cr ( $0.8603$ ,  $p=0.0130$ ), while in control children BMI correlated with right hippocampal NAA/mI ( $0.7417$ ,  $p=0.0352$ ). APOE 3 Mexico City parents showed significant positive correlations between BMI and the right hippocampus Cho/Cr ratio ( $0.6902$ ,  $p=0.0290$ ). MC APOE4 parents displayed negative correlations between BMI and right hippocampus mI/Cr ratio ( $-0.69$ ,  $p=0.02$ ), while positive correlations were seen with NAA/mI ratio ( $0.71$ ,  $p=0.01$ ).

## 4. Discussion

Seemingly healthy children with prenatal and lifetime exposures to fine particulate matter (PM<sub>2.5</sub>) and ozone above current US standards are showing a robust hippocampal decrease of NAA/Cr ratios, significantly affecting APOE4 carriers. These findings may constitute a spectral marker of early susceptibility to high air pollution exposures in a population carrying a gene with the strongest known genetic risk factor for Alzheimer's disease [18].

Significant hippocampal reduction of the NAA/Cr ratio, is a key finding in mild cognitive impairment and Alzheimer patients and has been observed in mouse models of AD [35-43]. N-acetyl aspartate (NAA) is highly concentrated in neurons, transferred to oligodendrocytes for use in myelination and myelin repair and involved in myelin lipid metabolism, neuronal osmoregulation and axon-glia signaling [44,45]. Since NAA is likely acting as a component of a neuron-oligodendrocyte metabolite trafficking system supporting oligodendrocyte metabolism during brain development and in response to brain injury [45], the reduced hippocampal NAA/Cr ratio in urban children has potential detrimental consequences.

The reduction of NAA/Cr in both gray and white matter is a key finding in AD patients and decreased levels in target areas predict future conversion of mild cognitive impairment to AD [46-51]. Murray et al., showed a decrease in the NAA/Cr ratio in the posterior cingulate gyrus is likely associated with loss of synapses and early pTau in subjects undergoing antemortem (1) H-MRS [43]. This is a very important association given we have shown pTau in Mexico City children and young adults, along with brain and intrathecal inflammation and dysregulated immune responses [7-9, 11,13,15,17]. Equally important, children in Mexico City have significant correlations between MRS altered metabolic ratios in key anatomical regions and targeted WISC-R subtests, all in keeping with involvement of AD target areas [46, 48, 50-55]. Overall, regardless of APOE genotype, MC children exhibit a significant decrease in the NAA/Cr ratio of the right frontal white matter, while APOE  $\epsilon 4$  children also exhibited decreases with age in pons NAA/Cr [14]. Notably, we reported MRS metabolic changes associated with odor deficits distinguishing MC APOE  $\epsilon 4$  v  $\epsilon 3$  children mainly related to frontal white matter NAA/Cr and hippocampal mI/Cr and NAA/Cr ratios [14].

Kjelvik [56] strongly supports the view that the hippocampus is specifically involved in higher-level processing of olfactory information, a concept discussed by Stäubli in 1984 [57]. Since anatomical areas correlating with odor identification [56] and hippocampal pathology have been linked to odor identification deficits, and episodic and semantic memory appear to support odor identification [56-58], hippocampal low NAA/Cr could impact odor identification in urban children [8]. Nutrition, energy balance, a proper crosstalk between the olfactory system and the hypothalamus, enjoyment of food, and their well-being under life threatening situations, could be compromised [8, 59, 60].

Since the left hippocampus NAA/Cr ratio was significantly different in APOE 4 children versus parents- the lowest values seen in parents-, we would be interested in exploring verbal/ general memory [41], and verbal fluency[37] in MC APOE 4 v APOE 3 parents. A complete cognitive evaluation and multivoxel MRI, along with volumetric MRI are warranted in these seemingly healthy parents.

The issue of the associations between adiposity and hippocampal-dependent and hippocampal-independent memory among prepubertal children and interactions of body mass index and apolipoprotein E  $\epsilon$ 4 allele on cognitive decline [23,24] are very relevant to urban children given the higher rates of obesity and the link between traffic-related air pollution and metabolic syndrome (MetS), obesity, hypertension, and diabetes mellitus (DM) [61-64]. Traffic pollution has been positively associated with growth in BMI in children aged 5-11 years in Southern California [63]. It is also critical that seemingly healthy normal weight Mexico City children are showing high levels of leptin, a powerful pro-inflammatory adipokine implicated in brain development, neuroplasticity, insulin resistance, obesity, type 2 diabetes and cardiovascular disease [65-68]. Complicating the air pollution scenario, Mexico City children showed dysregulation of feeding regulatory hormones including glucagon-like peptide-1, ghrelin, and glucagon [65], also involved in the modulation of reward processes, motivated behaviors and cognitive performances [69]. Thus, although the children in this study had mean BMI values within normal limits, diets high in saturated fatty acids (SFAs) [70], changes in nutritional habits [71], overweight and obesity will certainly be expected to worsen the brain metabolic responses with a significant impact on cognition[23, 72-77]. The significant positive correlations between BMI and the right hippocampus Cho/Cr ratio in APOE 3 Mexico City parents warrants further examination as Cho/Cr is a marker of membrane breakdown and has been associated with depression [78]. It is intriguing to interpret the Mexico City APOE 4 parents associations between BMI and ml/Cr and NAA/ml ratios in view of the provocative UK paper [25] contradicting the hypothesis that high BMI in middle age increases the risk of dementia. In fact, although our Mexico City APOE 4 parents are not obese, their BMI associations with selected hippocampal ratios are on the neuro protective side of the spectrum.

Notably, since pollution levels in MC have been sustained or worsened in the last 20 years [29], exposures of today's children and teenagers are truly lifelong and include significant exposures of their mothers during pregnancy. Longitudinal follow-up is required that examines the transition from childhood to adolescent stages on a range of cognitive, metabolic, volumetric and structural brain markers in relation to long term mental health outcomes, including cognitive and olfaction impairment and high risk for development of Alzheimer's disease. Significant decreases of NAA/Cr hippocampal ratios predominantly affecting urban APOE 4 children and middle age healthy carriers will warrant a close follow-up of MRS hippocampal changes due to its potential as a marker of loss of synapsis and hTau as described by Murray et al [43]. The early involvement of the right hippocampus is key, since we should be able to target specific neurocognitive deficits [79, 80].

## 5. Looking Forward, Limitations and Summary

We argue that sustained exposures to urban air pollution result in metabolic brain changes that may serve as very specific outcome measures for future longitudinal air pollution studies from early childhood, adolescence, and adulthood. The temporality of the metabolic involvement of the hippocampus in child/ parent pairs in the setting of air pollution exposures is critical to measure their cognitive detrimental impact. The combined effects of residency in a highly polluted city and APOE  $\epsilon$ 4 could lead to an acceleration of neurodegenerative changes and obligate us to have a broader focus on APOE  $\epsilon$ 4 [18, 21, 22], and to consider the impact of the body mass index on hippocampal responses in the equation of air pollution interactions impacting children's brains. Urban children's responses could provide new paths towards the unprecedented opportunity for AD prevention [81, 82].

We acknowledge our main limitations include the relatively small group of control subjects, the cross-sectionality of the study design and the need to concomitantly apply cognitive tools that establish the association with the hippocampal metabolic changes. Larger, longitudinal studies of children/parents pairs and neuropsychological data will be necessary to substantiate the significance of hippocampal NAA/Cr decreased ratios as spectral markers of early neurodegeneration in young urbanites. We also acknowledge that our efforts to minimize experimental confounds associated with the placement of MRS VOI although should greatly minimize inter-subject variability for the placement the VOI among subjects, it is plausible that percent of gray matter in the VOI may vary.

The identification of individuals at higher risk of developing Alzheimer's disease in the paediatric and young adult periods of brain development will allow us to implement multidimensional interventions having both broad impact and reach [83]. Protecting young urbanites from neural harmful effects of air pollution should be of pressing importance for public health.

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## FIGURE

Figure 1. A coronal image at the level of the medial temporal lobe and hippocampal regions for placement of MRS voxels. The representative voxel box in white (2cm X 2cm X 2cm) is drawn around the left hippocampus/medial temporal lobe to measure spectroscopic values of N-Acetylaspartate (NAA), Choline (Cho), Creatinine (Cr), and Myoinositol (mI).

