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The concentration of malondialdehyde in exhaled breath is influenced by air pollution exposure during physical activity in adolescents

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The concentration of malondialdehyde in exhaled breath is influenced by air pollution exposure during physical activity in adolescents

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2012

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An abstract of
A thesis submitted to the Faculty of the
Rollins School of Public Health of Emory University
in partial fulfillment of the requirements for the degree of
Master of Public Health
in Environmental Health
2014

Abstract

The concentration of malondialdehyde in exhaled breath is influenced by air pollution exposure during physical activity in adolescents
By Kaytna Thaker

Background: The health effects of air pollution have long been a concern, and numerous studies have found associations between air pollution exposure and adverse health effects. One of the key mechanisms through which air pollutants exert toxicity is believed to be oxidative stress. Malondialdehyde (MDA) is a relatively stable common lipid peroxidation product and has been quantified in a variety of biological matrices, including exhaled breath condensate (EBC). ***Objectives:*** To determine the presence of an association between increased levels of MDA in EBC and increased concentrations of measured air pollutants within this study population of adolescents. ***Methods:*** Student athletes between the ages of 14 and 18 were recruited from sports practices at two metro-Atlanta high schools. Prior to, and immediately following sports team practices, samples of EBC were collected from each subject. Air quality measurements were recorded for the duration of team practices. MDA concentrations were measured in EBC using High Performance Liquid Chromatographic Mass Spectrometry following derivatization with 2,4-dinitrophenylhydrazine. Single pollutant models using linear regression were used to assess associations between percent change in MDA and pollutants of interest. ***Results:*** A non-significant decrease in MDA levels was observed when comparing pre and post-practice MDA concentration among all subject, however, a significant difference was observed in post-practice MDA levels between urban and suburban schools. A significant difference in the mean percent change in MDA levels among subjects participating in indoor and outdoor sports. A statistically significant association was found between particle number count and percent change in MDA levels and a borderline significant association was found between ozone and percent change in MDA levels. Controlling for gender, a statistically significant association was found between ozone and particle number count and percent change in MDA level. ***Discussion:*** MDA levels increased during sports team practices. Independent of exposure, outdoor sports are associated with increased percent change in MDA levels when compared to indoor sports. Although a significant difference was observed in the mean post-practice MDA concentration among urban and suburban schools, there was no significant difference in the overall percent change in MDA levels between the two schools.

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Acknowledgements

I would like to thank Dana Barr, Barry Ryan, Elizabeth Marder, Priya Kewada, Anastasia Lopukin, and Gabrielle Fanning-Dowdell for their contributions to this work.

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INTRODUCTION

Background

Ambient air pollution is a complex mixture of gases and particles that arise from a variety of sources. Anthropogenic sources of air pollution include stationary sources such as fossil fuel burning power plants and industrial facilities as well as mobile sources such as cars, trucks, and construction and agricultural equipment. The health effects of air pollution have been a concern for several decades, as evidenced by air quality standards mandated by the U.S. EPA and the European Union's Air Quality Limit Values. Exposure to ambient air pollution has been linked to numerous adverse health effects in populations throughout the world. Both short and long term studies have found associations between exposure to air pollutants and increased cardiovascular and respiratory morbidity and mortality, even at low levels of exposure.[1,2] Recently air pollution has come under increased scrutiny, with the International Agency for Research on Cancer classifying outdoor air pollution as a carcinogen. Additionally, traffic-related pollutants including ozone and particulate matter have been associated with asthma exacerbation and airway hyperresponsiveness among children and cardiovascular disease mortality among adults. [3-6]

Children and Air Pollution

Children in particular may be more susceptible to harmful effects of air pollutants compared to adults, as their lungs are not yet fully developed. Children may have greater exposures than adults, and children generally spend a greater proportion of time spend a lot of time outdoors and engage in physical activities that increase their breathing rate.[7-9] Although lung development begins prenatally, with alveoli forming between fetal weeks

32-36, this process continues well into childhood.[10] Structural and morphological features of the child lung begin to resemble those of an adult lung between ages 3 and 4. Using cell culture and animal models including non-human primates, toxicological studies have demonstrated exposure to air pollution alters the morphological construction of the airways, ultimately altering their functional capacity.[11-14] Researchers have observed in animal models that differentiation of the lung epithelium can be altered by insult at a very young age and because repair mechanisms are not fully developed, these alterations persist.[15]

Epidemiological studies indicate that exposure to air pollutants are associated with decreased lung growth. The Children's Health Study conducted in California from 1993 to 2001 observed deficits in lung function and decreased lung development correlated with $PM_{2.5}$ and elemental carbon levels.[16] However this study only evaluated lung development between the ages of 10 to 18 and did not consider deficits in development that may have resulted from exposure to air pollutants in early childhood. A sub-study within this cohort was conducted to evaluate the association between playing team sports and development of asthma during a 5 year follow up period.[17] Researchers found that development of physician-diagnosed asthma was associated with high ambient levels of ozone. These results were consistent among both children with previous history of asthma symptoms and those who had not previously exhibited symptoms of asthma such as wheezing. A prospective cohort study conducted among Austria schoolchildren from 1994-1997 observed decreases in pulmonary function associated with PM_{10}, and Jedrychowski et al. also found consistent associations between air pollutants and decreased lung growth among preadolescent children in Krakow, Poland.[18,19]

Health Effects of Air Pollution

Oxidative stress is believed to be one of the key underlying features of air pollutant toxicity.[20] Oxidative stress is generally described as an imbalance between free radicals (often reactive oxygen species or reactive nitrogen species) and antioxidants; excess oxidative products can damage DNA, form protein adducts, and damage lipid membranes leading to the formation of lipid peroxidation products.[21,22] Although oxidative stress can target a variety of cellular components, lipids are the most involved class of biomolecule due to the abundance of lipids in the cell membrane and various other cellular constituents.[23]

Using cell and animal models, toxicological studies report air pollution induced oxidative stress and additional human exposure studies have found indications of airway inflammation following exposure to traffic-related air pollution in both healthy and asthmatic subjects.[24-28] Epidemiological studies have demonstrated that short term increases in pollutants such as fine particulate matter ($PM_{2.5}$), ozone, and elemental or black carbon are associated with increased airway oxidative stress in children and adolescents.[29,30] Vossoughi et al. found evidence of increased airway and systemic inflammation in a cohort following long-term exposure to traffic and industrial air pollution, and numerous respiratory diseases are associated with airway inflammation, including asthma, chronic bronchitis, and chronic obstructive pulmonary disease.[31,32]

Although air pollutants appear to exacerbate airway inflammation, the extent to which oxidative stress contributes to air pollution induced toxicity and airway inflammation remains unclear.[20,33,34] As such, a variety of techniques have been developed to quantify biomarkers of airway inflammation and oxidative stress.

Noninvasive techniques such as the collection of exhaled breath condensate have proven useful. Exhaled breath condensate (EBC) is a fluid formed by cooling exhaled air. Although this fluid consists mostly of condensed water vapor, it also contains respiratory fluid droplets released from the lung epithelial lining fluid, which contain traces of volatile and non-volatile solutes that can be recovered in EBC samples.[35] Biomolecules including aldehydes, leukotrienes, 8-isoprostane, prostaglandins, hydrogen peroxide, nitric oxide-derived products, and hydrogen ions can be measured in EBC.[36]

Malondialdehyde (MDA) is a relatively stable common lipid peroxidation product and has been quantified in a variety of biological matrices, including EBC. [37-41] Several studies have reported increased levels of MDA along with increased levels of air pollutants. Barregard et al. reported higher levels of MDA following exposure to wood smoke, supporting the hypothesis that particulate air pollution causes oxidative stress and distal pulmonary inflammation.[42] Among asthmatic schoolchildren in Mexico City, Romieu et al. reported associations between levels of MDA in EBC and levels of ozone and particulate matter.[39] Additionally, MDA levels were inversely associated with lung function. A study conducted during the 2008 Beijing Olympics found associations between MDA levels in healthy individuals and both single pollutants and multi-pollutant combinations, indicating the oxidative stress effects of air pollution.[38]

Aims

Through the collection and analysis of health measurements and air quality data, this analysis aims to demonstrate an association between increased levels of MDA in EBC and increased levels of air pollutants. EBC samples will be analyzed to determine the levels of MDA present in each sample. MDA levels will be compared with air

pollutant data to determine the presence of an association between increased levels of MDA in EBC and increased concentrations of measured air pollutants within this study population of adolescents.

METHODS

Study Population and Enrollment

The Study of Air Pollution and Physical Activity (SAPPA) seeks to determine the effect of physical activity upon air pollution exposure. The study primary investigator is Roby Greenwald, PhD (Dept. of Environmental Health), and it was funded by NIH grant # 1K25ES020355-01A1.The study protocol was reviewed and approved by Emory University's Institutional Review Board.

Demographic and health information was gathered through a questionnaire. The questionnaire included questions about date of birth, sex, race/ethnicity, height and weight, smoking status, and history of asthma.

Student athletes between the ages of 14 and 18 were recruited from sports practices at two metro-Atlanta high schools. Sports included cross country, football, soccer, basketball, track & field, and marching band. Study consent was obtained from the subjects, or in the case of subjects under the age of 18, from the subject's parent/guardian. Prior to, and immediately following sports team practices, biological samples and health measurements were obtained from each subject. Air quality measurements were recorded for the duration of team practices and constitute exposure. Measured levels of pollutants are representative of ambient concentrations to which subjects were exposed. Inhaled dose represents the amount of pollutants taken into the

lungs and varies among subjects depending on breathing rate and lung capacity. For the purposes of this study, ambient concentrations were used as model inputs.

Subjects who self-identified as a current smoker were excluded from the study. A total of 179 subjects have participated in the study to date. For the purposes of this analysis, a subset of 58 subjects was randomly selected for analysis.

EBC Sample Collection

Samples of exhaled breath condensate (EBC) were collected from each subject prior to and following exposure using an R-tube® collection device with an aluminum sleeve chilled to -80° Celsius. Subjects breathed through the R-tube® for approximately 10-15 minutes, or until at least 1 mL of condensate was produced. An aliquot of 245 μL of EBC was added to 5 μL of butylated hydroxytoluene (BHT), to prevent further oxidation of MDA in the EBC fluid. Subsequently, EBC samples were stored at -80° C until analysis.

MDA Standard Solution Preparation

MDA standard solutions were prepared by dissolving malondialdehyde tetrabutylammonium salt in acetonitrile to yield a 10 mM stock solution. This stock solution was diluted with deionized H_2O to create yield a linear calibration solutions with concentrations ranging from 0.001 μM to 1.0 μM (Appendix B, Figure 2).

EBC Sample Preparation and Derivitization

Malondialdehyde (MDA) concentrations were measured in EBC using an adaptation of the method described by Chen et al.[43] First, EBC samples were thawed and vortexed. Then, a 50 μL aliquot of each EBC sample was mixed with 25 μL of an internal standard solution (1 μM of d_2-MDA) and with 20 μL of 1M HCl. The EBC samples were

derivitized using 25 μL of a saturated 2,4-dinitrophenylhydrazine (DNPH) solution to form a DNPH-MDA adduct under an incubation temperature of 37 ° Celsius for 70 mins. Blank samples, consisted of deionized H_2O, and 7 standard calibrants were prepared concurrently with the EBC samples, using the same procedure, for each batch of sample analysis.

EBC Sample Quantification

EBC samples containing a DNPH-MDA adduct were separated and analyzed using a 6190 High Performance Liquid Chromatographic Mass Spectrometric (LC-MS) system (Agilent, USA). The analytical column was a C18 column, 150 × 4.6 mm I.D., particle size 5 μm (Phenomenex, USA). Column compartment temperature was set at 45° C. Mobile phases consisted of acetonitrile (containing 0.1% formic acid, v/v) and deionized water (containing 0.1% formic acid, v/v). The instrument flow rate was set at 0.8 mL/min. with an injection volume was 100μL.

Mass spectrometric analysis of the DNPH-MDA adduct was performed under a positive Electrospray Ionization (EIS), using a Single Ion Monitoring (SIM) technique. An *m/z* of 235 was monitored for native DNPH-MDA adduct, while an *m/z* of 237 was monitored for its label isotope analogue. Mass spectrometric parameters were as follows: 120 fragmentor, 594 dwell time, 360°C sheath gas temperature, 325°C drying gas temperature, 10 L/min sheath gas flow, 5 L/min drying gas flow, 35 psi nebulizer pressure, 4000V capillary voltage, and 800V nozzle voltage. See Appendix A for detailed method information.

Analytical Method Performance

The method limit of quantification is defined as the concentration of the lowest standard solution, which contained 0.001 μM MDA. The accuracy of this method was assessed by taking subtracting the expected concentration of each standard from the observed concentration and averaging these values. Using these parameters, the accuracy of this method is 102%. The method precision, reported as an average relative standard deviation (RSD), was below 15%. Note that all analytical parameters reported here were in compliance with the requirements set by the FDA.

Data Analysis

Analyses were performed using SAS version 9.3. Exploratory analyses were performed and values for each variable. Univariate summaries were used to analyze continuous variables and relative frequency tables were used to analyze categorical variables and check for implausible or missing values. Exposures were recorded as mean concentration of each pollutant of interest. Single pollutant models using linear regression were used to assess associations between percent change in MDA and pollutants of interest.

RESULTS

Summary statistics

A subset of 58 subjects was randomly selected for this analysis. Subjects were recruited at various high school sports team practices. Table 1 presents the characteristics of the study subjects. Prior to and immediately following sports team practices, each subject completed a series of health measurements and provided samples of exhaled breath condensate. Subjects with an MDA % change greater than 3 standard deviations

from the mean were removed from analysis. Results are based on the remaining 52 subjects. Mean MDA concentrations before and after sports team practices and MDA % change are presented in Table 2.

Table 1: Study subject characteristics

			Gender		Sport	
School	**Total Subjects**	**Mean Age (yr)**	**Male**	**Female**	**Indoor**	**Outdoor**
Urban	34	16.88 ± 1.14	33	1	19	15
Suburban	18	17.03 ± 1.16	6	12	6	12

Table 2: Mean pre and post-practice MDA concentrations (standard deviation) and MDA % change

	Urban School	**Suburban School**	**All Subjects**
Pre-practice (μM)	0.0144 (0.0228)	0.0158 (0.0127)	0.0149 (0.0198)
Post-practice (μM)	0.0120 (0.0066)	0.0268 (0.0299)	0.0171 (0.0194)
Percent Change (%)	42.85 (84.00)	81.06 (109.75)	56.07 (94.43)

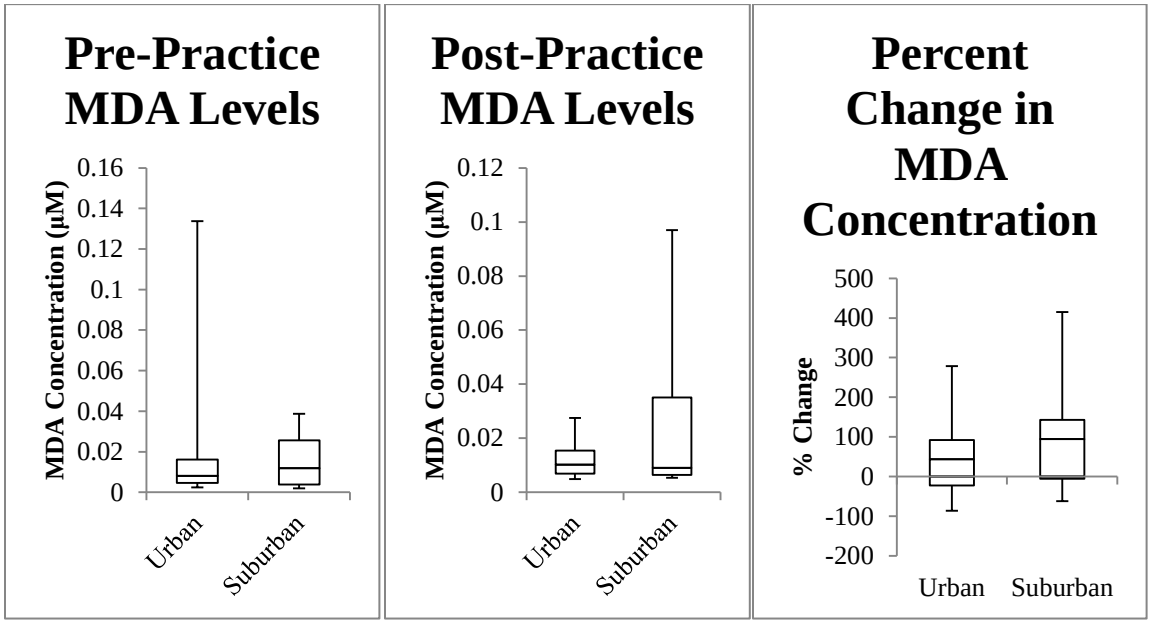
Differences in pre vs. post-practice MDA levels between subjects from urban and suburban schools

Using the mean MDA concentrations as indicated in Table 2, a non-significant decrease in MDA levels was observed when comparing post and pre-practice MDA concentration among all subjects (-0.0022 μM, p=0.4569). A similar non-significant increase was observed for subjects from the urban school (0.0024 μM, p-=0.5113), however a significant decrease in post vs. pre-practice MDA levels was observed among subjects from the suburban school (-0.0110 μM, p=0.0358). These differences do not take into account air pollutant levels.

Differences in mean MDA levels between subjects from urban and suburban schools

The mean pre and post-practice MDA levels and percent change in MDA among subjects at urban and suburban schools were compared using one-way ANOVA (Figure 1). No significant difference was observed in pre-practice MDA levels (p=0.8064) or percent change in MDA (p=0.1673), however a significant difference was observed in post-practice MDA levels between urban and suburban schools (p=0.0076).

Figure 1: Differences in mean MDA levels between subjects from urban and suburban schools



Differences in pre vs. post-practice MDA levels between subjects participating in indoor and outdoor sports

Using the mean MDA concentrations as indicated in Table 3, a non-significant decrease in MDA levels was observed when comparing post and pre-practice MDA concentration among all subjects (-0.0022 μM, p=0.4569). A non-significant increase was observed for subjects participating in indoor sports (0.0019 μM, p=0.7614), however a borderline significant decrease in post vs. pre-practice MDA levels was observed among

subjects participating in outdoor sports (-0.0026 μM, p=0.0594). These differences do not take into account air pollutant levels.

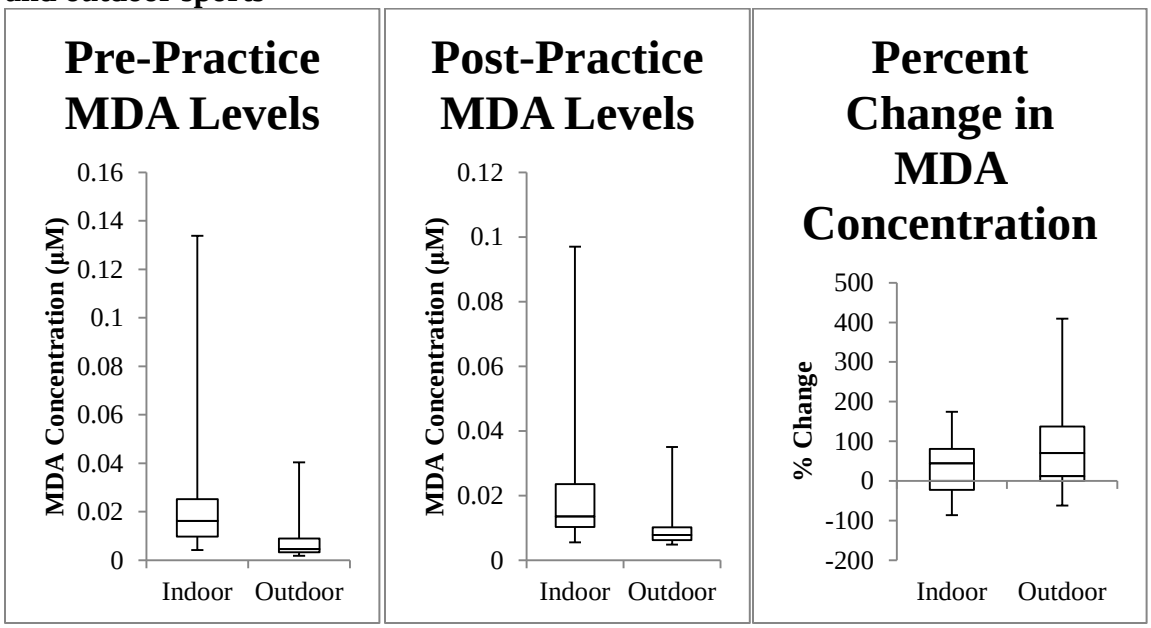
Table 3: Mean pre and post-practice MDA concentrations (standard deviation) and MDA % change

	Indoor Sports	Outdoor Sports	All Subjects
Pre-practice (μM)	0.0220 (0.0254)	0.0084 (0.0090)	0.0149 (0.0198)
Post-practice (μM)	0.0238 (0.0253)	0.0110 (0.0081)	0.0171 (0.0194)
Percent Change (%)	24.8 (70.15)	85.03 (05.54)	56.07 (94.43)

Differences in mean MDA levels between subjects participating in indoor and outdoor sports

The mean pre and post-practice MDA levels and percent change in MDA among subjects participating in indoor and outdoor sports were compared using one-way ANOVA (Figure 2). A significant difference was observed in pre-practice MDA levels (p=0.012) and post-practice MDA levels (p=0.0153). Additionally, a significant difference in the percent change in MDA levels (p=0.02).

Figure 2: Differences in mean MDA levels between subjects participating in indoor and outdoor sports



Pollutants

Ozone, black carbon, and particle number count were recorded during sports team practices. Mean concentrations of each pollutant are presented in Table 4. Both ozone and black carbon mean concentrations are greater at the suburban school compared to the urban school. Ozone, black carbon, and particle number count are all substantially greater among outdoor sports when compared to indoor sports.

Table 4: Mean pollutant concentrations (standard deviation) at urban and suburban schools and indoor and outdoor sports

	School		**Sport**	
Pollutant	Urban School	Suburban School	Indoor Sports	Outdoor Sports
Ozone (ppb)	20 (18)	33 (22)	5 (0)	42 (11)
Black carbon (µg/m^3)	0.20 (0.22)	0.29 (0.41)	0.013 (0.009)	0.33 (0.32)
Particle number count (#/cm^3)	9434 (5848)	2906 (598)	5364 (1544)	9854 (7366)

Associations between air pollutants and percent change in MDA levels

Single pollutant linear regression models were used to assess associations between air pollutants and percent change in MDA levels. As indicated in Table 5, no statistically significant association was found black carbon and percent change in MDA levels. A statistically significant association was found between particle number count and percent change in MDA levels and a borderline significant association was found between ozone and percent change in MDA levels. Using the interquartile range (IQR), an increase of 37 ppb in ozone levels is associated with a 45.9% change in MDA levels and an increase of 3365 particles/cm^3 is associated with a 17.16% increase in MDA levels. When multi-pollutant linear regression models were considered, no statistically significant associations at the α=0.05 level were found between pollutants and percent change in MDA level.

Table 5: Single pollutant linear regression models examining the relationship between ozone, black carbon, and particle number count and percent change in MDA levels

Pollutant	Parameter estimate	Standard error	P-value
Ozone (ppb)	1.24	0.63	0.0563
Black carbon ($\mu g/m^3$)	-31.04	54.84	0.5749
Particle number count ($\#/cm^3$)	0.0051	0.0023	0.0350

When sex and age are included as additional predictors a statistically significant association was found between ozone and particle number count and percent change in MDA level. Because age was not a significant predictor of percent change in MDA levels, it was removed from the models. Gender was coded as 0/1, with females (coded as 1) exhibiting a larger percent change. Controlling for gender, a 37 ppb increase in ozone

is associated with a 47.4% increase in percent change of MDA levels and an increase of 3365 particles/cm^3 is associated with a 26.25% increase in MDA levels.

Equation 1: Single pollutant linear regression model controlling for sex

$$\text{Percent change MDA} = \beta_0 + \beta_1 * \text{Pollutant concentration} + \beta_2 * \text{Gender}$$

Table 6: Single pollutant linear regression models including sex as additional predictors of percent change in MDA level controlling for sex and age

Pollutant	Parameter Estimate	Standard Error	P-value
Ozone	1.28	0.59	0.035
Sex	81.21	27.39	0.0047
Black carbon	-17.53	54.67	0.750
Sex	57.69	38.53	0.143
Particle number count	0.0078	0.002	0.002
Sex	87.72	32.18	0.0064

When considering multi-pollutant linear regression models including age and sex as additional predictors, no statistically significant association at the α=0.05 level was found between pollutants and percent change in MDA level.

DISCUSSION

The mean percent change in MDA levels among all subjects was 56% and when stratifying by urban and suburban school, the mean percent change in MDA levels was 43% and 81% respectively. These values indicate that MDA levels increased during sports team practices which constituted the exposure period. Independent of exposure, outdoor sports are associated with increased percent change in MDA levels when compared to indoor sports. Several studies have reported increased MDA levels following exposure to traffic related air pollution, and these results are in agreement.[38,39] Although a significant difference was observed in the mean post-practice MDA

concentration among urban and suburban schools, there was no significant difference in the overall percent change in MDA levels between the two schools.

Previous studies have found increased levels of MDA in EBC following exposure to traffic-related pollutants including ozone, black carbon, and $PM_{2.5}$ among asthmatic and healthy subjects. [38,39] Additionally, exposure to traffic-related pollutants has been associated with decreased lung function, asthma exacerbation, and airway inflammation.[4,16,44] In this study, particle number count was found to be significantly associated with percent change in MDA levels. Although not statistically significant, the results of this study suggest that ozone may be associated with percent change in MDA levels. Black carbon was not found to be significantly associated with percent change in MDA levels.

For the purposes of this study, ambient concentrations were used as model inputs. It is for this reason that exposure may not necessarily reflect inhaled dose. Additionally, exposure levels for ozone, particle number count, and black carbon were relatively low compared to other studies. This is due to the fact that concentrations of air pollutants in indoor environments are typically lower than outdoor concentrations. Also, levels of measured pollutants tend to increase during the summer months in Atlanta. However, when compared to previous summers, the summer of 2013 was unusually cool and rainy. The relatively small number of subjects included in this analysis is also a limitation. Ongoing analysis of additional samples will improve our ability to determine the magnitude of the associations between pollutants and percent change in MDA levels.

When controlling for age and gender, both particle number count and ozone were found to be significantly associated with percent change in MDA levels. Age does not

appear to have a significant impact upon percent change in MDA levels, however based on the results of this analysis, gender appears to be a significant predictor of the percent change in MDA levels. This association between gender and percent change in MDA levels has not been observed in previous studies. Further analyses are needed to determine if this association is merely a function of the small sample size of this analysis. The significant associations between particle number count and percent change in MDA levels are in agreement with previous studies. However the results of this analysis failed to find a significant association between ozone and black carbon and percent change in MDA levels.

The influence of physical activity upon MDA levels remains unclear.[45,46] The results of this study indicate that MDA levels increased over the course of sports team practices, presumably while subjects were engaging in physical activity. A greater percent change in MDA levels was observed among subjects participating in outdoor sports (85%) compared to subjects participating in indoor sports (25%). These results appear to indicate that independent of exposure to air pollutants, engaging in physical activity corresponds to an increase in MDA levels.

Limitations

Due to the noninvasive nature of collection, EBC is a promising matrix in which a variety of biomolecules can be quantified. However, variability exists in the collection of EBC. Although aluminum sleeves are originally chilled to -80° Celsius, over time the temperature of sleeves increases. Ambient temperature has been found to impact the pH of EBC samples which in turn can affect the electrochemical properties and reaction

kinetics of the EBC.[47] The R-tube® collection devices are designed in a manner so as to limit salivary contamination of EBC fluid; however the method used in this analysis cannot determine if samples of EBC have been contaminated with saliva. It is necessary to test for high concentrations of phosphate or amylase to determine if samples are contaminated.

The high degree of variability in measurement is a limitation of the EBC matrix. Because water vapor is the major constituent of EBC, the concentration of analytes, including malondialdehyde, exhibit significant interindividual variations as well as intraindividual variations.[48] Numerous studies have compared levels of MDA across individual subjects, however in order to do so more accurately, further research is necessary to determine an accurate method of quantifying the dilution factor of EBC samples.[49] EBC was collected at specific time points (immediately before and after sports team practices) in order to best capture variations in MDA concentrations due to exposure however further research is necessary to determine the optimal collection time point.

CONCLUSIONS

The data from this study indicate that exposure to particulate matter and ozone is associated with increased MDA levels although further research is needed to determine the true magnitude of this association. This study focuses on children, as they may be more susceptible to adverse health effects of air pollution exposure compared to adults. Additionally, the associations observed in this study provide further insight into the pollutants most likely to induce oxidative stress and airway inflammation. Increased

levels of MDA are indicative of increased oxidative stress and airway inflammation which may be one mechanism through which traffic-related air pollutants exert toxicity.

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FIGURES AND TABLES

Table 1: Study subject characteristics

School	Mean Age (yr)	Gender (M/F)	Sport (Indoor/Outdoor)	Total Subjects
Urban	16.88 ± 1.14	33/1	19/15	34
Suburban	17.03 ± 1.16	6/12	6/12	18

Table 2: Mean pre and post-practice MDA concentrations (standard deviation) and MDA % change

	Urban School	Suburban School	All Subjects
Pre-practice (μM)	0.0144 (0.0228)	0.0158 (0.0127)	0.0149 (0.0198)
Post-practice (μM)	0.0120 (0.0066)	0.0268 (0.0299)	0.0171 (0.0194)
Percent Change (%)	42.85 (84.00)	81.06 (109.75)	56.07 (94.43)

Table 3: Mean pre and post-practice MDA concentrations (standard deviation) and MDA % change

	Indoor Sports	Outdoor Sports	All Subjects
Pre-practice (μM)	0.0220 (0.0254)	0.0084 (0.0090)	0.0149 (0.0198)
Post-practice (μM)	0.0238 (0.0253)	0.0110 (0.0081)	0.0171 (0.0194)
Percent Change (%)	24.8 (70.15)	85.03 (05.54)	56.07 (94.43)

Table 4: Mean pollutant concentrations (standard deviation) at urban and suburban schools and indoor and outdoor sports

	School		Sport	
Pollutant	Urban School	Suburban School	Indoor Sports	Outdoor Sports
Ozone (ppb)	20 (18)	33 (22)	5 (0)	42 (11)
Black carbon ($\mu g/m^3$)	0.20 (0.22)	0.29 (0.41)	0.013 (0.009)	0.33 (0.32)
Particle number count ($\#/cm^3$)	9434 (5848)	2906 (598)	5364 (1544)	9854 (7366)

Table 5: Single pollutant linear regression models examining the relationship between ozone, black carbon, and particle number count and percent change in MDA levels

Pollutant	Parameter estimate	Standard error	P-value
Ozone (ppb)	1.24	0.63	0.0563
Black carbon (μg/m^3)	-31.04	54.84	0.5749
Particle number count (#/cm^3)	0.0051	0.0023	0.0350

Table 6: Single pollutant linear regression models including sex as additional predictors of percent change in MDA level controlling for sex and age

Pollutant	Parameter Estimate	Standard Error	P-value
Ozone	1.28	0.59	0.035
Sex	81.21	27.39	0.0047
Black carbon	-17.53	54.67	0.750
Sex	57.69	38.53	0.143
Particle number count	0.0078	0.002	0.002
Sex	87.72	32.18	0.0064

Figure 1: Differences in mean MDA levels between subjects from urban and suburban schools

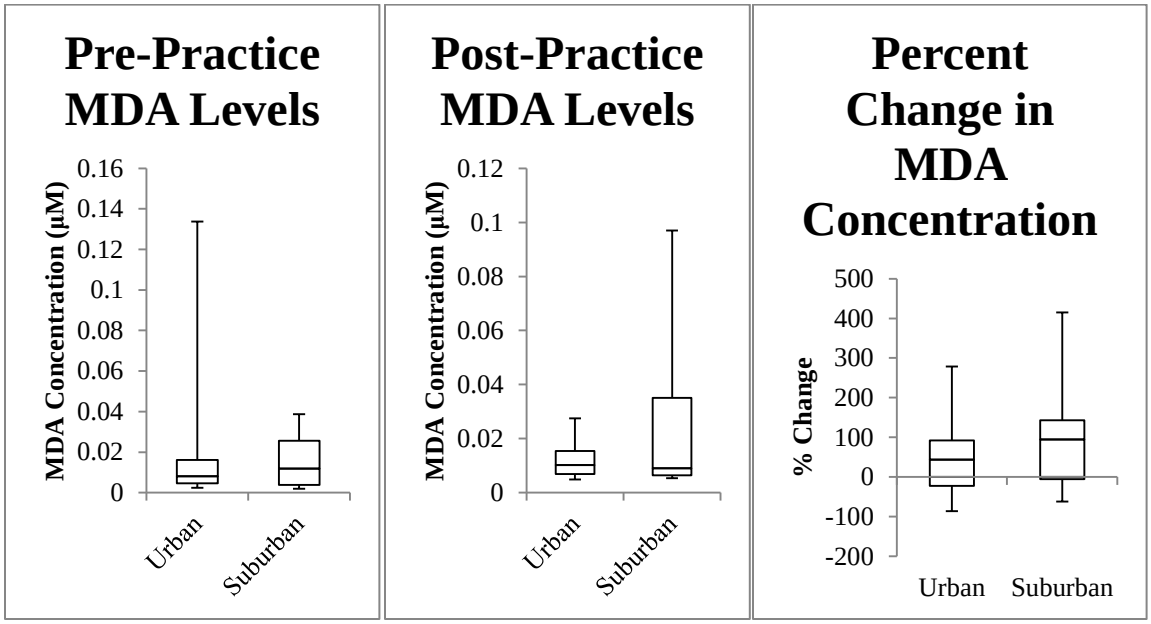
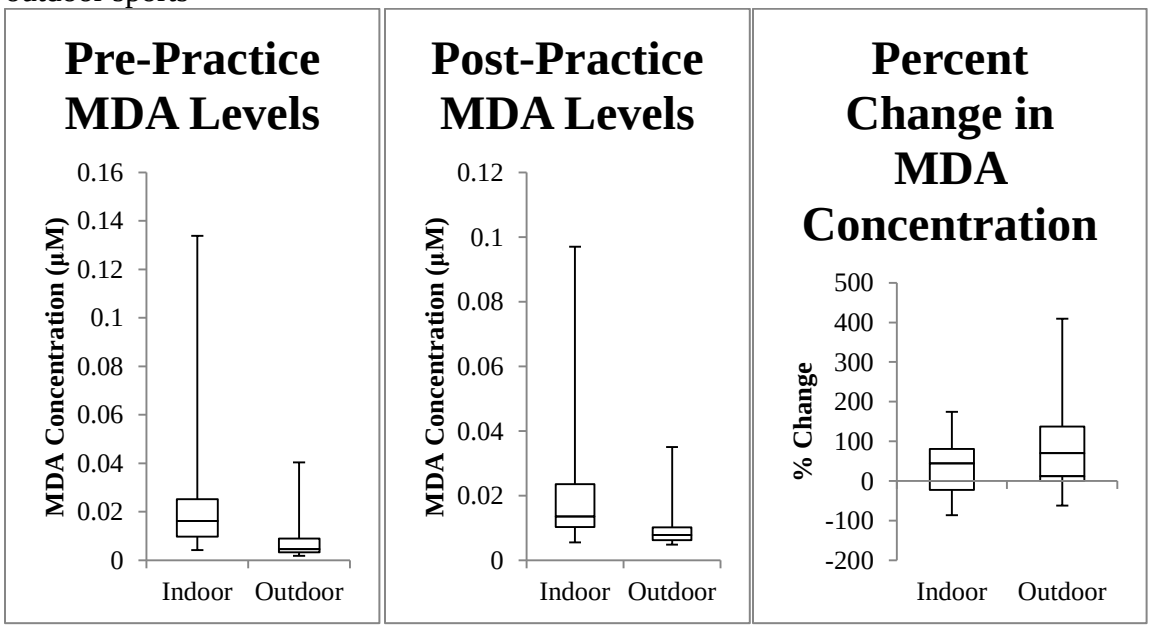


Figure 2: Differences in mean MDA levels between subjects participating in indoor and outdoor sports



Appendix A: Full LC-MS Analytical Method

Method Information

Method: C:\Chem32\1\METHODS\MDA-DNPH_F2.M
Modified: 3/5/2014 at 2:21:11 PM

MS final method for MDA-DNPH: Positive SIM mode

Method Audit Trail

Operator :
Date : 3/5/2014 12:50:53 PM
Change Info: This method was created at 3/5/2014 12:50:53 PM and based on method C:\Chem32\1\METHODS\MDA-DNPH_F.M

Operator :
Date : 3/5/2014 12:51:07 PM
Change Info: Method saved. User comment: "Test Meth 030414"

Operator :
Date : 3/5/2014 12:58:55 PM
Change Info: Method saved. User comment: ""

Operator :
Date : 3/5/2014 2:21:11 PM
Change Info: Method saved. User comment: ""

Run Time Checklist

Pre-Run Cmd/Macro: off

Data Acquisition: on

Standard Data Analysis: off

Customized Data Analysis: off

Save GLP Data: off

Post-Run Cmd/Macro: off

Save Method with Data: off

==========
==========
Binary Pump
==========
==========

Binary Pump (G4220A)
==========

Flow: 0.100 ml/min
Use Solvent Types: Yes
Stroke Mode: Synchronized
Low Pressure Limit: 0.00 bar
High Pressure Limit: 550.00 bar
Max. Flow Ramp Up: 100.000 ml/min²
Max. Flow Ramp Down: 100.000 ml/min²
Expected Mixer: No check

Stroke A
Automatic Stroke Calculation A: Yes

Compress A
Compressibility Mode A: Compressibility Value Set
Compressibility A: 45 10e-6/bar

Compress B
Compressibility Mode B: Compressibility Value Set
Compressibility B: 75 10e-6/bar

Stop Time
Stoptime Mode: Time set
Stoptime: 10.00 min

Post Time
Posttime Mode: Time set
Posttime: 1.00 min

Timetable
Time Function Parameter

0.00 Change Flow Flow: 0.8 ml/min
1.00 Change Solvent Com. Solvent composition A: 35.0 % B:65.0 %
5.00 Change Solvent Com. Solvent composition A: 40.0 % B:60.0 %
5.50 Change Solvent Com. Solvent composition A: 0.0 % B:100.0 %

8.00 Change Solvent Com. Solvent composition A: 0.0 % B:100.0 %
8.50 Change Solvent Com. Solvent composition A: 50.0 % B:50.0 %
10.00Change Solvent Com. Solvent composition A: 50.0 % B:50.0 %

Solvent Composition

Channel	Ch. 1 Solv.	Name 1
A	100.0 % Water V.02	0.1% NH4OH
B	100.0 % Acetonitrile V.02	0.1% Formic Acid

Ch2 Solv.	Name 2	Selected	Used
100.0 % Water V.02	0.1% Formic Acid	Ch. 2	Yes
100.0 % Methanol V.02	0.1%Acetic Acid	Ch. 1	Yes

Percent %
50.0
50.0

Instrument Curves
Store Pressure: Yes
Store Flow: Yes
Store Solvent Ratio A: Yes
Store Solvent Ratio B: Yes

==========

HiP Sampler

==========
======

HiP Sampler (G4226A)
==========

Post Time
Posttime Mode: Off

Auxiliary
Draw Speed: 100.0 µl/min
Eject Speed: 200.0 µl/min
Draw Position Offset: 0.0 mm
Wait Time After Drawing: 2.0 s
Sample Flush Out Factor: 5.0
Vial/Well bottom sensing: No

Injection
Injection Mode: Standard injection
Injection Volume: 20.00 µL

High throughput
Valve To Bypass After Injection Enabled: No

Overlapped Injection
Enable Overlapped Injection: No

Valve Switching
Valve Movements: 0

Valve Switch Time 1
Switch Time 1 Enabled: No

Valve Switch Time 2
Switch Time 2 Enabled: No

Valve Switch Time 3
Switch Time 3 Enabled: No

Valve Switch Time 4
Switch Time 4 Enabled: No

Stop Time
Stoptime Mode: As pump/No limit

Timetable

HiP Sampler (G4226A)
==========

Function	Parameter
Repeat	Repeat 4 time(s)
Draw	Draw 20 µL from sample with default speed using default offset
Wait	Wait 0.02 min
Eject	Eject default volume to seat with default speed using default offset
End Repeat	End Repeat
Draw	Draw 20 µL from sample with default speed using default offset
Wait	Wait 0.02 min
Inject	Inject

==========

Column Comp.
==========

Column Comp. (G1316C)
==========

Ready when front door open: Yes

Left Temperature Control
Temperature Control Mode: Temperature Set
Temperature: 45.00 °C

Enable Analysis Left Temperature

Enable Analysis Left Temperature On: Yes
Enable Analysis Left Temperature Value: 0.80 °C

Right Temperature Control
Right temperature Control Mode: Combined

Enable Analysis Right Temperature
Enable Analysis Right Temperature On: Yes
Enable Analysis Right Temperature Value: 0.80 °C

Stop Time
Stoptime Mode: As pump/injector

Post Time
Posttime Mode: Off

Timetable

Instrument Curves
Store Left Temperature: No
Store Right Temperature: No

==========
Mass Spectrometer Detector
==========

General Information
------- -----------

Use MSD : Enabled
Tune File : atunes.tun
StopTime : No Limit
Time Filter : Enabled
Data Storage : Condensed
Peakwidth : 0.20 min
Fast Scan : Disabled

Fast Scan Data Reconstruction: Disabled
Polarity Switch Delay : 20 ms
Ionization Switch Delay : 50 ms

Signals

[Signal 1]

Not Active

[Signal 2]

Ionization Mode : AJS-ES
Polarity : Positive
Fragmentor Ramp : Not Applicable
Percent Cycle Time : 100.00 %
Sim On Target Mass : Disabled

Sim Parameters

Time (min)	Group Name	SIM Ion	Frag-mentor	Gain EMV	Actual Dwell	Compound Name	ISTD
2.00	Target	235.00	120	5.0	594	MDA-DNPH	0
		237.00	120		594	MDA-DNPH	1
6.00	Not Active						

[Signal 3]

Not Active

[Signal 4]

Not Active

Spray Chamber

[MSZones]

SGas Temp : 360 C maximum 360 C
Gas Temp : 325 C maximum 350 C
DryingGas : 5.0 l/min maximum 13.0 l/min
SGas Flow : 10.0 l/min maximum 12.0 l/min
Neb Pres : 35 psig maximum 60 psig

VCap (Positive) : 4000 V
VCap (Negative) : 4000 V
NozzleVolt (Positive) : 800 V
NozzleVolt (Negative) : 800 V

[Time Table]

Time Table is empty.

END OF MS ACQUISITION PARAMETERS

==========
FIA Series
==========

FIA Series in this Method : Disabled

Time Setting
Time between Injections : 0.15 min
Injection Loop Flush Time : 0.17 min

==========
Estimated Sample Purity Calculation
==========

General
Estimated Sample Purity Calculation is disabled.

The Data Analysis Parameters of the used Method are :

==========
Integration Events
==========

Non signal specific Integration Events

Event	Value
Tangent Skim Mode	Standard
Baseline Correction	Advanced
Tail Peak Skim Height Ratio	0.000
Front Peak Skim Height Ratio	0.000
Skim Valley Ratio	20.000
Peak to Valley Ratio	500.000

Default Integration Event Table "Event"

Event	Value	Time
Initial Slope Sensitivity	1.000	Initial
Initial Peak Width	0.020	Initial
Initial Area Reject	1.000	Initial
Initial Height Reject	1.700	Initial
Initial Shoulders	OFF	Initial

Detector Default Integration Event Table "Event_DAD"

Event	Value	Time
Initial Slope Sensitivity	5.000	Initial
Initial Peak Width	0.020	Initial
Initial Area Reject	5.000	Initial
Initial Height Reject	1.000	Initial
Initial Shoulders	OFF	Initial

Detector Default Integration Event Table "Event_ADC"

Event	Value	Time
Initial Slope Sensitivity	1.000	Initial
Initial Peak Width	0.020	Initial
Initial Area Reject	1.000	Initial
Initial Height Reject	1.700	Initial
Initial Shoulders	OFF	Initial

Detector Default Integration Event Table "Event_FLD"

Event	Value	Time
Initial Slope Sensitivity	1.000	Initial
Initial Peak Width	0.020	Initial
Initial Area Reject	1.000	Initial
Initial Height Reject	1.700	Initial
Initial Shoulders	OFF	Initial

Detector Default Integration Event Table "Event_MSD"

Event	Value	Time
Initial Slope Sensitivity	10000.000	Initial
Initial Peak Width	0.020	Initial
Initial Area Reject	1000.000	Initial
Initial Height Reject	100.000	Initial
Initial Shoulders	OFF	Initial

Signal Specific Integration Event Table "Event_MSD1TIC"

Event	Value	Time
Initial Slope Sensitivity	100000.000	Initial
Initial Peak Width	0.020	Initial
Initial Area Reject	10000.000	Initial
Initial Height Reject	1000.000	Initial
Initial Shoulders	OFF	Initial

Signal Specific Integration Event Table "Event_MSD1SPC"

Event	Value	Time
Initial Slope Sensitivity(Full Scan)	1.000	Initial
Initial Peak Width(Full Scan)	0.250	Initial
Initial Slope Sensitivity(Cond. Scan/SIM)	0.100	Initial
Initial Peak Width(Cond. Scan/SIM)	0.050	Initial
Initial Area Reject	0.000	Initial
Initial Height Reject	5.000	Initial
Initial Shoulders	OFF	Initial

Detector Default Integration Event Table "Event_VWD"

Event	Value	Time
Initial Slope Sensitivity	1.000	Initial
Initial Peak Width	0.020	Initial
Initial Area Reject	1.000	Initial
Initial Height Reject	1.700	Initial
Initial Shoulders	OFF	Initial

Detector Default Integration Event Table "Event_ECD"

Event	Value	Time
Initial Slope Sensitivity	1.000	Initial
Initial Peak Width	0.020	Initial
Initial Area Reject	1.000	Initial
Initial Height Reject	1.700	Initial
Initial Shoulders	OFF	Initial

Detector Default Integration Event Table "Event_MWD"

Event	Value	Time
Initial Slope Sensitivity	1.000	Initial

Initial Peak Width	0.020	Initial
Initial Area Reject	1.000	Initial
Initial Height Reject	1.700	Initial
Initial Shoulders	OFF	Initial

Apply Method's Manual Integration Events: No

==========

Specify Report

==========

Calculate: Area Percent
Use Multiplier & Dilution Factor with ISTDs

Use Sample Data from Data File
Destination: Screen
Quantitative Results sorted by: Signal
Report Style: Short
Sample info on each page: No
Add Chromatogram Output: Yes
Chromatogram Output: Portrait
Size in Time direction: 100 % of Page
Size in Response direction: 40 % of Page
Uncalibrated Peaks: Report with Calibrated Peaks

==========

Signal Options

==========

Include: Axes, Retention Times, Baselines, Tick Marks
Font: Arial, Size: 8

Ranges: Full
Multi Chromatograms: Separated, Each in full Scale

==========

Calibration Table

==========

Calib. Data Modified :

Rel. Reference Window : 5.000 %
Abs. Reference Window : 0.000 min
Rel. Non-ref. Window : 5.000 %
Abs. Non-ref. Window : 0.000 min
Uncalibrated Peaks : not reported
Partial Calibration : Yes, identified peaks are recalibrated
Correct All Ret. Times: No, only for identified peaks

Curve Type : Linear
Origin : Included
Weight : Equal

Recalibration Settings:
Average Response : Average all calibrations
Average Retention Time: Floating Average New 75%

Calibration Report Options :
Printout of recalibrations within a sequence:
Calibration Table after Recalibration
Normal Report after Recalibration
If the sequence is done with bracketing:
Results of first cycle (ending previous bracket)

==========

Peak Sum Table

==========

No Entries in table

==========

==========

Sample related custom fields

```
================================================================
=========

Custom Field                Type    Mand.  Default Value
----------------------------------------------------------------
None defined

================================================================
=========
                Compound related custom fields
================================================================
=========

Custom Field                Type    Mand.  Default Value
----------------------------------------------------------------
None defined
```

Appendix B: MDA Calibration Curve