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Exhaled breath malondialdehyde as a biomarker of effect of exposure to traffic-related air pollution

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Bachelor of Science
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2010

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An abstract of
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Abstract

Exhaled breath malondialdehyde as a biomarker of effect of exposure to traffic-related air pollution

By Weiwei Li

Background: Traffic-related air pollution induces lung inflammation and oxidative stress. Noninvasive measurement of biomarker such as malondialdehyde (MDA) in exhaled breath condensate (EBC) is particularly useful for evaluating the role of oxidative stress and inflammation in acute responses to exposures that occur in vehicles. ***Objectives:*** To examine whether short-term exposure to traffic-related air pollutants affects MDA levels in EBC as a biomarker of oxidative stress. ***Methods:*** 21 healthy subjects and 21 asthmatic subjects are recruited in this study. All subjects operated vehicles for two hours during the morning rush period on scripted roadways in the Atlanta region. Each vehicle is equipped with instrumentation to monitor concentrations of air pollutants. EBC samples are collected pre and post-exposure by cooling down the exhaled breath in a refrigerated collecting device. The concentrations of MDA in EBC were measured by high-performance liquid chromatography (HPLC) fluorescence detection after the EBC samples were derived with thiobarbituric acid (TBA). Statistic approaches used to analyze the changes of MDA levels include repeated measures ANOVA and t-test. Mixed effect regression models are used to analyze the associations between in-vehicle pollutants (BC and PAHs) and the changes of MDA levels during the prescribed commute. ***Results****:* EBC-MDA levels increased immediately after exposure to the 2-hour commuting. From one hour after the commute, MDA levels decreased to constant values similar as the MDA levels one day before the commute. The changes of MDA levels for asthmatic subjects are more significant than healthy subjects. Moreover, there are several significant associations between the in-vehicle pollutants (especially PAHs) and the changes of MDA levels. ***Discussion:*** MDA in EBC is a useful biomarker of effect of exposure to traffic-related air pollutants. This marker of oxidative stress is a promising tool in future studies of traffic-related air pollution.

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INTRODUCTION

Exposure to ambient air pollution has been shown to have negative effect on human health. Numerous epidemiological studies have associated exposure to ambient air pollutants with a range of adverse respiratory and cardiovascular health effects in different populations around the globe. Ambient air pollution studies have focused primarily on particulate matter (PM) and ozone, which are two of the U.S. Environmental Protection Agency's "criteria pollutants".[1] More recently, traffic-related air pollutants have been linked to increased respiratory and cardiovascular morbidity and mortality, including asthma incidence and exacerbation, and hospitalization and death due to myocardial infarction.[2,3,4] Moreover, exposure to vehicle-associated PM has also been concerned particularly from a public health perspective.

Relatively few studies of animals or humans with exposure to realistic traffic conditions are available. In studies of animals, collected particles such as road dust and diesel exhaust particles have been reported to cause pulmonary and systemic oxidative stress and inflammation.[5] In addition, a few studies have used experimental or quasi-experimental approaches to study human adverse health effects to roadside traffic[6] or in-vehicle exposure while in traffic.[7,8] A bus trip study shows that fine particle exposures resulted in increased levels of FE(NO) in elderly adults, suggestive of increased airway inflammation.[7] Additionally, another recent study of asthmatic adults in London found that the lung function of subjects walking for two hours along a street primarily utilized by diesel vehicles declined to a much greater degree than the lung function of those walking through a park area in central London.[6] Particularly, this effect was more pronounced for subjects with moderate asthma than for those with mild asthma.

Moreover, lung function differs a lot although the difference in air quality between the two exposures was fairly small ($PM_{2.5}$ mass of 12 $\mu g/m^3$ for the park vs. 28 $\mu g/m^3$ for the street). This study suggests that short-term exposures to even moderate amounts of traffic-related air pollution can negatively influence lung function.

Oxidative stress appears to play an important role in the toxicity of air pollutants.[9] Oxidative stress is defined as an impaired balance between free radical production and antioxidant capacity resulting in excess oxidative products.[10] The generation of reactive oxygen species can thereafter cause oxidative damage to DNA, proteins, or lipids in the body.[11] Oxidative stress has been reported to be induced by air pollution in animal studies.[12] Epidemiologic studies conducted in urban areas have also demonstrated that oxidative stress was associated with environmental air pollutants in bus drivers.[13] Particles containing polycyclic aromatic hydrocarbons (PAHs), which are tightly adsorbed to the black carbon (BC) surface, have been shown to contribute to cellular oxidative damage, particularly to mitochondria.[14, 15] However, relatively few studies to date have directly measured acute respiratory or systemic oxidative stress in humans following exposure to traffic or traffic-related pollutants.[9]

Among the biological targets of oxidative stress, membrane lipids are the most commonly involved class of biomolecules. Lipid peroxides are recognized as being more and more important in signal transduction for a number of events in the inflammatory response[16]. Inflammation plays an important pathophysiological role in respiratory diseases such as asthma and chronic obstructive pulmonary disease (COPD).[17, 18] Therefore, monitoring of airway inflammation is useful in the follow up of patients with respiratory diseases, and for guiding pharmacological therapy.

The complexity of near-roadway exposure presents a major challenge to assess human responses to traffic-related air pollution under realistic conditions. Noninvasive techniques for measuring oxidative stress and inflammation in human airways can help to translate the results of in vitro and animal studies to human experience, and facilitate greater understanding of how widespread exposures to traffic affect public health.

Recently, attention has focused on exhaled breath condensate (EBC) as a noninvasive method to monitor inflammatory and oxidative stress of lower respiratory tract.[19] Noninvasive measurement of biomarkers in EBC is particularly useful for evaluating the role of oxidative stress and inflammation in acute responses to exposures that occur in vehicles or during near-roadway activities. EBC is a fluid formed by cooling exhaled air. Several biomarkers are measureable in EBC, including aldehydes, H_2O_2, adenosine, isoprostanes, leukotrienes and cytokines.[20] The analysis of EBC samples has been given great weight as a noninvasive methodology of studying respiratory diseases.[21]

Products of lipid peroxidation are promising markers of oxidative stress and inflammaion in EBC, as well as in blood and urine.[9] Malondialdehyde (MDA) is one of the major final products of lipid peroxidation, which is a free-radical-mediated degradative process. In this progress, polyunsaturated fatty acids in cell membranes are preferentially affected, leading to the formation of lipid hydoperoxides.[22] Previously, Several studies have reported an increase of peripheral blood MDA in smokers,[23] in nonsmoking bus drivers,[24] and in female students after environmental exposure to air pollutants.[25] Another study found statistically significant increases in urinary MDA levels with ambient PM concentrations in children, which indicates the association between exposure of PM air pollution and oxidative stress in schoolchildren.[26] In addition, MDA

in EBC has been measured in several studies. In the study of Aldehydes in exhaled breath condensate of patients with chronic obstructive pulmonary disease, MDA has been measured in EBC. The result shows that MDA is increased in patients with COPD compared with healthy subjects.[27] Recently, Romieu and colleagues reported an increase in EBC-MDA associated with increased PM and ozone among children with asthma in Mexico City.[28] In this study, MDA in EBC was measured as a biomarker of exposure to traffic-related pollution. The increase in MDA was inversely correlated with lung function. Furthermore, there was a significant trend of increasing MDA in EBC with increasing traffic counts at local intersections. While in my study, MDA in EBC was evaluated as a biomarker of oxidative stress, which is considered as a health effect. The commute itself is considered as exposure. The concentrations of pollutants such as BC and PAH are used as parameters of the exposure.

MDA in EBC has been measured in relatively few studies and only few of them include large number of subjects. In my study, MDA in EBC is measured and compared in 42 subjects with 77 commutes in order to explore the usefulness of MDA in EBC as a marker of effect of exposure to traffic-related air pollutants.

METHODS

Subjects

A total of 42 subjects (21 physician-diagnosed adult asthmatics and 21 healthy adults) are recruited for participation in this study. Subjects are recruited with flyers and word of mouth at Emory University and Centers for Disease Control and Prevention (CDC). The main characteristics of the subjects in our study are shown in table1. The

mean age is 35.6 (95% CI: 31.9, 39.3) for healthy subjects and 30.8 (95% CI: 26.4, 35.2) for asthmatic subjects. Each subject is asked to complete a scripted commute during two different seasons of the year. However, some subjects didn't attend to the second commute for personal reason, and the total commute number is 38 for healthy subjects and 39 for asthmatic subjects.

Table 1. Main characteristics of the subjects under study

	Subject No	Age	Gender (M/F)	Total commute No
Healthy subjects	21	35.6±3.7	12/9	38
Asthmatic subjects	21	30.8±4.4	8/13	39

Study design

In-vehicle exposures are characterized for all subjects operating their personal vehicles on roadways in the Atlanta region. Subjects are directed to follow a scripted commute lasting approximately two hours during the morning rush hour period. Each route begins at either Emory University, except one subject began at the subject's residence, and finishes at Emory University. Further, each subject is asked to complete a scripted commute during two different seasons of the year.

To monitor the concentrations of PM, BC and PAHs in the vehicle cabin, each vehicle was equipped with instrumentation. The inlets for all instruments were situated as close to the driver's breathing zone as possible, and all pump exhausts were routed to the exterior of a rear window.

EBC collection

Exhaled breath condensate (EBC) was collected by cooling down the exhaled breath in a chilled collecting device (Figure 1). Subject breathed through a cold tube for

about 10 – 15 minutes to produce about 1 mL of condensed water vapor one day before scheduled tests, immediately prior to the start of the commute and immediately following the commute and again at 1-hour intervals for 3 hours. The timing of EBC sampling is presented in table 2.

Table 2. Timing of EBC sampling

Sample ID	Time	
Pre1	Before commute	One day before commute
Pre		Immediately prior to the start of the commute
Post	After commute	Immediately following the commute
Post1		One hour after commute
Post2		Two hours after commute
Post3		Three hours after commute

In the collecting device (Figure 1), the condensation chamber is a disposable polypropylene tube that is connected to an exhalation valve that also serves as a syringe-style plunger to pull fluid off the condenser walls.[29] The refrigerating system consists of an aluminium-cooling sleeve, which is placed over the disposable polypropilene tube. The temperature of the cooling sleeve can be chosen by the investigator. [29] In my study, the temperature is -80 °C. The R Tube is particularly suitable when collection of several samples a day is required, as in our study.

Since EBC samples need to be stored for analysis, conditions of storage of samples in the laboratory should be kept as similar as possible. In our study, EBC samples were immediately stored at -80 °C until analysis.

Figure 1. RTube (Respiratory Research, Charlottsville, Virginia, USA). The RTube is a portable EBC collecting system that consists of a disposable polypropylene condensation chamber with an exhalation valve, which also serves as a syringe-style plunger to pull fluid off the condenser walls. Cooling is achieved by placing an aluminium cooling sleeve over the disposable polypropylene tube. [30]

Malondialdehyde Analysis in EBC

Malondialdehyde (MDA) concentrations were measured in EBC samples according to the method described by Lärstad et al.[22] Samples were derivatized with thiobarbituric acid (TBA) to form a pink condensation product, which can be detected by high-performance liquid chromatography (HPLC) fluorescence detection.

According to Lärstad et al, TBA reagent was prepared as a 25 mM TBA solution in 0.30 M phosphoric acid–KOH buffer, pH 3.5. Since TBA is hardly soluble, the

solvation of TBA was accomplished by mixing in a sonicating water bath at 40 °C. Fresh solution was prepared daily. The stock solution of MDA (5.0 mM) was prepared by dissolving 40 mg of MDA tetrabutylammonium salt in 25 ml of water-ethanol (60:40, v/v). The working standards were prepared by diluting the stock solution with water. The stock solution was stored at +7 °C and is stable for 1 month. Working standards were prepared daily.

MDA samples were prepared by adding 50 μl aliquots of breath condensate to 450 μl TBA regent. The samples were vortex-mixed for 1 s and derivatisation was performed in a heat plot at 95 °C for 60 minutes. Then the samples were cooled in ice for 5 minutes, and then allowed to recover at room temperature for 40 minutes. The prepared samples were vortex-mixed for 1 s before analysis.

Analyses were performed using a high-performance liquid chromatography (HPLC) connected to a fluorescence detector (Shimadzu, Japan), equipped with a C_{18} column, 150 × 4.6 mm I.D., particle size 5 μm (Phenomenex, USA).). An aliquot of 400 μl was injected into the HPLC system. The mobile phase was composed by acetonitrile: 20 mM potassium phosphate buffer, pH 6.8 (20: 80, v/v). The flow rate was 1.3mL/min. Excitation and emission wavelengths were of 532 and 553 nm, respectively. Chromatograms were registered and peak areas determined using Shimadzu's LCsolution software.

The determination of MDA in samples was preformed with a three-point calibration curve based on water blank measurement and two measurements at higher concentration levels (0.05 μM and 0.1 μM). Since the calibration curve may change with the influences of daily prepared TBA and the running buffer, calibration points are

obtained daily to calculate MDA levels of the samples analyzed in that day. The linearity of the calibration curve was checked using a quality control measurement at a concentration level of 0.5 μM.

Data Analysis

My specific hypotheses in this study include the following 3. I used different method to test these hypotheses.

Hypothesis 1:

H_0: There is no difference among MDA levels in EBC collected at any of the 6 time points pre and post commute.

Ha: There are differences among MDA levels in EBC collected at some of the 6 time points pre and post commute.

For this hypothesis, the 2-hour commute is considered as exposure. I used repeated measures ANOVA to compare MDA levels in EBC sampled from the same subject one day before the commute (Pre1), immediately prior to the start of the commute (Pre), immediately following the commute (Post), and at 1-hour intervals for 3 hours following the commute (Post1, Post2, Post3). For each subject, the EBC-MDA samples at these 6 time points are dependent. Thus repeated measures ANOVA method is used to compare these 6 dependent variables measured in the same subjects. The groups compared with MDA levels immediately following the commute include 1) one day before the commute (Post vs. Pre1); 2) immediately prior to the start of the commute (Post vs. Pre); 3) one hour after the commute (Post vs. Post1); 4) two hours after the commute (Post vs. Post2) and 5) two hours after the commute (Post vs. Post3). In

addition, The MDA levels of 1-hour intervals after the commute for 3 hours are all compared with the MDA levels immediately prior to the start of the commute (Post1 vs. Pre, Post2 vs. Pre and Post3 vs. Pre). The repeated measures ANOVA method were used to analyze the interindividual difference of MDA levels for all subjects, healthy subject and asthmatic subjects respectively. The results after testing this hypothesis would indicate whether the MDA levels in EBC increased after exposure to the 2-hour commute.

Hypothesis 2:

H_0: There is no difference among MDA levels between healthy subjects and asthmatic subjects at any of the 6 time points pre and post commute.

Ha: There are differences among MDA levels between healthy subjects and asthmatic subjects at some of the 6 time points pre and post commute.

Similar as the first hypothesis, exposure is the 2-hour commute. I used t-test to compare the differences in MDA levels between these two independent groups of subjects - healthy subjects and asthmatic subjects. The levels of MDA in EBC sampled at each of the 6 time points, including one day before commute (Pre1), immediately before the start of the commute (Pre), immediately following the commute (Post) and 1-hour interval for 3 hours after the commute (Post1, Post2 and Post3) were all analyzed respectively to test the differences between healthy subjects and asthmatic subjects. The results from this analysis would indicate whether health status affect MDA levels.

Hypothesis 3:

H_0: The change in EBC-MDA levels between pre and post-exposure is not related to the pollutant levels.

Ha: The change in EBC-MDA levels between pre and post-exposure is related to the pollutant levels.

For hypothesis 3, the exposures are in-vehicle pollutants (BC and PAHs), which are different from hypothesis 1 and 2. Mixed effect regression models are used to test the hypothesis. The change of MDA levels between each subject's pre-commute and post-commute measurements is used as an outcome variable in a mixed effects model. The model treats the in-vehicle values as fixed effects to establish baseline associations. The results from this mixed effect regression models could show the association between in-vehicle pollutants (BC and PAHs) and the changes of MDA levels during the prescribed commute.

RESULTS

Summary statistics

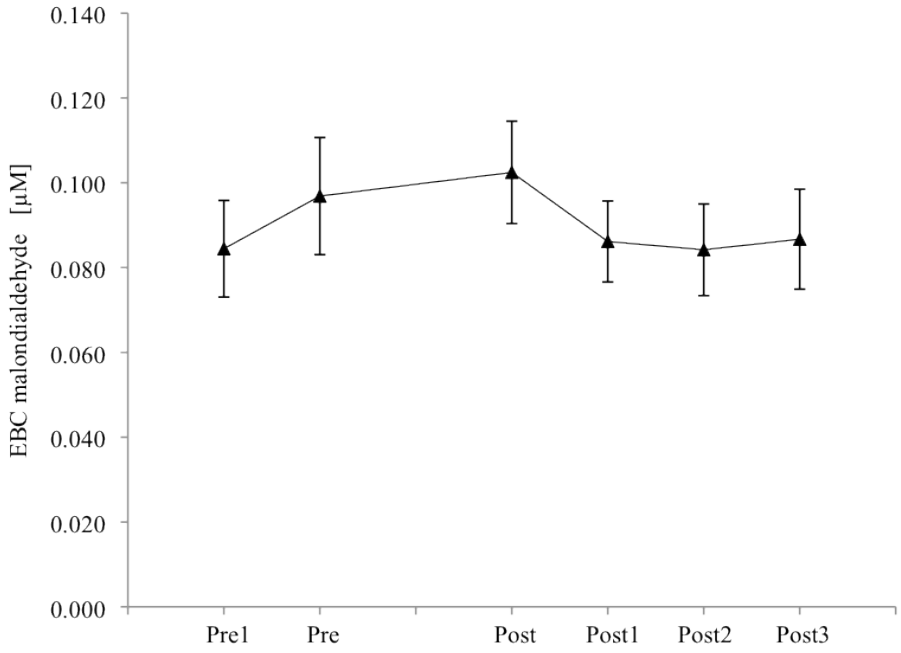
The mean and 95% confidence intervals of EBC-MDA levels are presented in table 3. Results are based on 77 commutes (38 from healthy subjects and 39 from asthmatic subjects).

Table 3. MDA levels (μM) before and after commute

	Before		**After Commute**			
Sample ID	Pre1	Pre	Post	Post1	Post2	Post3
All Subjects	0.084, (0.073-0.096)	0.097 (0.083-0.111)	0.102 (0.090-0.115)	0.086 (0.077-0.096)	0.084 (0.073-0.095)	0.087 (0.075-0.098)
Healthy Subjects	0.079 (0.065-0.094)	0.090 (0.072-0.108)	0.092 (0.078-0.105)	0.081 (0.067-0.096)	0.082 (0.064-0.101)	0.081 (0.064-0.098)
Asthmatic Subjects	0.088 (0.071-0.106)	0.103 (0.082-0.123)	0.112 (0.093-0.132)	0.090 (0.077-0.103)	0.086 (0.073-0.099)	0.091 (0.075-0.108)

The MDA levels changes through the 6 time points before and after the commute. As shown in figure 2, the changes of MDA levels are quite different when analyzing for all subjects or analyzing for healthy subjects or asthmatic subjects only.

(A)



(B)

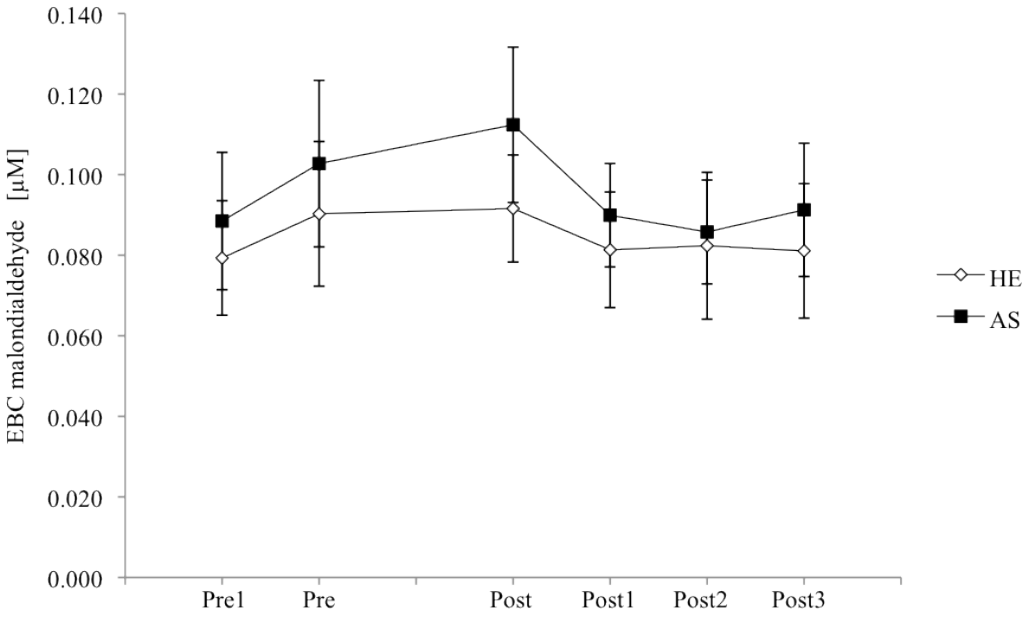


Figure 2. Malondialdehyde (MDA) in exhaled breath condensate levels before and after the exposure to 2-hour commuting. The figure shows the MDA levels (mean and 95% confidence intervals) one day before commute (Pre1), immediately prior to the start of the commute (Pre), immediately following the commute (Post), and at 1-hour intervals for 3 hours following the commute (Post1, Post2, Post3). The results are shown for MDA levels from all subjects (A), and MDA levels from healthy or asthmatic subjects (B).

A nonsignificant increase of MDA levels immediately after the commute (Post vs. Pre) was noted based on all subjects (5.8%, 0.102 (0.090-0.115) vs. 0.097 (0.083-0.111) μM, p=0.4874). While analyzing MDA changes for healthy and asthmatic subjects respectively, there is an even slighter nonsignificant increase for healthy subjects (1.5%, 0.092 (0.078-0.105) vs. 0.090 (0.072-0.108) μM, p=0.8951) but a little higher nonsignificant increase for asthmatic subjects (9.4%, 0.112 (0.093-0.132) vs. 0.103 (0.082-0.123) μM, p=0.4825).

When comparing MDA levels in EBC sampled immediately after commute with in EBC sampled one day before commute (Post vs. Pre1), a significant increase was

noted based on all subjects (21.3%, 0.102 (0.090-0.115) vs. 0.084, (0.073-0.096) μM, p=0.0252). The change for asthmatic subjects is bigger (27.0%, 0.112 (0.093-0.132) vs. 0.088 (0.071-0.106) μM, p=0.0322). But there is no significant change for healthy subjects (15.5%, 0.092 (0.078-0.105) vs. 0.079 (0.065-0.094) μM, p=0.3135).

From one hour after the commute (Post1, Post2 and Post3), the MDA levels kept to be quite consistent and similar as the MDA levels one day before the commute (Pre1). For all subjects, the MDA levels at those 4 time points are all around 0.085 μM (Pre1: 0.084 (0.073-0.096) μM; Post1: 0.086 (0.077-0.096) μM; Post2: 0.084 (0.073-0.095) μM; Post3: 0.087 (0.075-0.098) μM). For healthy subjects, the MDA levels at those 4 time points are all around 0.081 μM (Pre1: 0.079 (0.065-0.094) μM; Post1: 0.081 (0.067-0.096) μM; Post2: 0.081 (0.064-0.098) μM; Post3: 0.081 (0.064-0.098) μM). For asthmatic subjects, the MDA levels at those 4 time points are all around 0.088 μM (Pre1: 0.088 (0.071-0.106) μM; Post1: 0.090 (0.077-0.103) μM; Post2: 0.086 (0.073-0.099) μM; Post3: 0.091 (0.075-0.108) μM).

Differences of MDA levels between healthy subjects and asthmatic subjects

Noticing the different changes of MDA levels between healthy subjects and asthmatic subjects, I used t-test to compare MDA levels at each of the 6 time points (Pre1, Pre, Post, Post1, Post2 and Post3) between these two subgroups. T-test results are presented in table 4.

Table 4. T-statistics and p-values for the t-test of mean MDA levels between healthy subjects and asthmatic subjects.

	t Value	Pr > |t|
Pre1	0.78	0.4377
Pre	0.88	0.3809
Post	1.74	0.0867**
Post1	0.87	0.3862
Post2	0.31	0.7604
Post3	0.84	0.4038

** Not significant, but suggestive

The results show that, at the 0.05 significance level, there is no significant difference of mean MDA levels in EBC sampled at each of the 6 time points between healthy subjects and asthmatic subjects. It indicates that, at each time point (one day before commute, immediately before the start of the commute, immediately following the commute and 1-hour interval for 3 hours after the commute), the means of MDA levels could be considered equal for healthy subjects and asthmatic subjects. However, although the difference of mean MDA levels immediately following the commute (Post) between healthy subjects and asthmatic subjects is not significant (p-value=0.0867), it's suggestive. The distributions of MDA levels at Post time point for healthy subjects and asthmatic subjects show differences between the two for most subjects. The results suggests that the MDA levels for healthy subjects could not be considered as equal with the MDA levels for asthmatic subjects immediately following the commute.

Changes of MDA levels for healthy subjects and asthmatic subjects

Being aware of the difference of MDA levels in EBC sampled immediately following the commute between healthy subjects and asthmatic subjects, I analyzed the

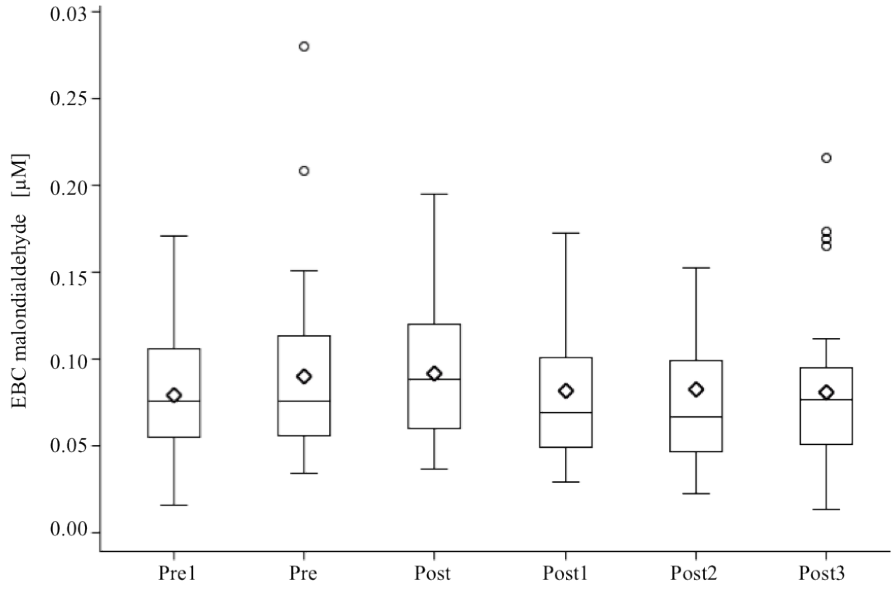
changes of MDA levels between pre and post-commute for healthy subjects and asthmatic subjects respectively. Repeated Measures Analysis of variance (ANOVA) method was used to test the interindividual differences of those dependent MDA levels. MDA levels in EBC sampled in the same subject one day before commute (Pre1), immediately prior to the start of the commute (Pre), immediately following the commute (Post), and at 1-hour intervals for 3 hours following the commute (Post1, Post2, Post3) were compared with each other among healthy subjects and asthmatic subjects respectively. The statistics of the Repeated Measures ANOVA analysis are presented in table 5.

Table 5. The statistics of the Repeated measures ANOVA analysis of mean MDA levels before and after commute for healthy subjects or asthmatic subjects.

Subjects	Parameter	t Value	Pr > |t|
Healthy	Post vs. Pre1	1.06	0.3135
	Post vs. Pre	0.12	0.8951
	Post1 vs. Pre	-0.86	0.3916
	Post2 vs. Pre	-0.81	0.4188
	Post3 vs. Pre	-0.77	0.4440
	Post vs. Post1	0.89	0.3250
	Post vs. Post2	0.82	0.3480
	Post vs. Post3	0.92	0.3712
Asthmatic	Post vs. Pre1	2.08	0.0322*
	Post vs. Pre	0.45	0.4825
	Post1 vs. Pre	-1.35	0.1784
	Post2 vs. Pre	-1.71	0.0894**
	Post3 vs. Pre	-1.09	0.2764
	Post vs. Post1	2.03	0.0420*
	Post vs. Post2	2.37	0.0173*
	Post vs. Post3	1.58	0.0749**

* Significant change at the 0.05 level ** Not significant, but suggestive

The results show that there is no significant change of MDA levels in effect of the commute for healthy subjects. While for asthmatic subjects, there are several changes significant, including the changes in EBC-MDA levels between immediately following the commute and 1) one day before the commute (Post vs. Pre1, p-value=0.0322); 2) one hour after the commute (Post vs. Post1, p-value=0.0420); 3) two hours after the commute (Post vs. Post2, p-value=0.0173). Also, from the Boxplot of MDA levels before and after commutes in Figure 3 A, we can easily tell that the changes for healthy subjects are quite flat; while for asthmatic subject, those three changes are significant at the 0.05 significance level (Figure 3. B). In addition, the differences of mean MDA levels between two hours after the commute and immediately prior to the start of the commute (Post2 vs. Pre, p-value=0.0894), and between immediately following the commute and three hours after the commute (Post vs. Post3, p-value=0.0749) are both suggestive for asthmatic subjects although they are not significant at the 0.05 significance level (Figure 3. B).



(A)

(B)

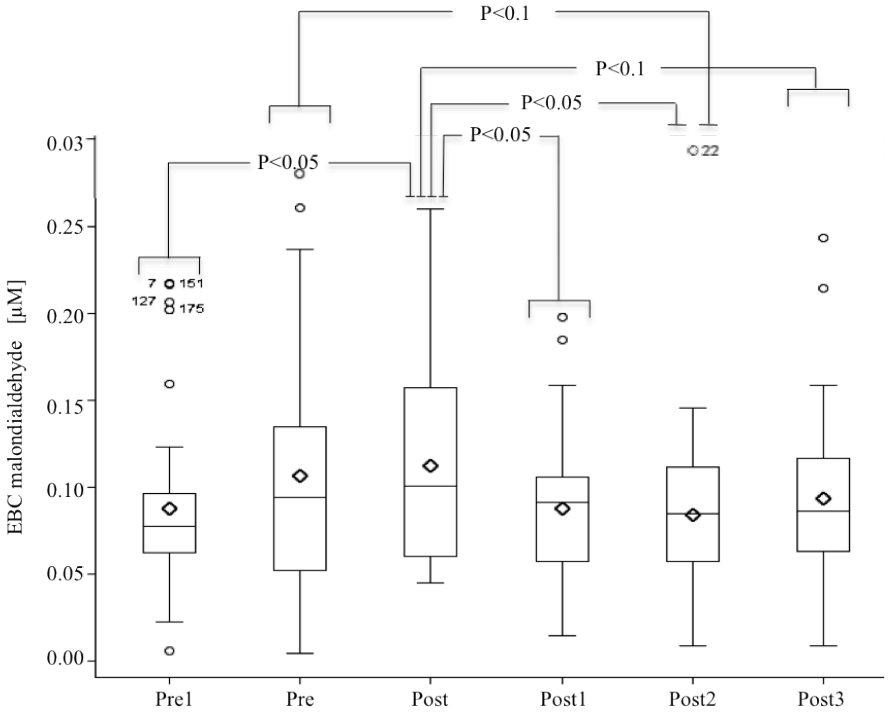


Figure 3. Boxplot of Malondialdehyde (MDA) in exhaled breath condensate levels before and after commutes. The figure shows the MDA levels (mean, 25th, 50th and 75th percentile, minimum value and maximum value) one day before commute (Pre1), immediately prior to the start of the commute (Pre), immediately following the commute

(Post), and at 1-hour intervals for 3 hours following the commute (Post1, Post2, Post3). The results are shown for MDA levels from healthy subjects (A) and asthmatic subjects (B) respectively.

Changes of MDA levels in effect of exposure to the commute

To study the effects of exposure to 2-hour commuting, intraindividual changes of MDA levels one day before the commute (Pre1), immediately after the commute (post), one hour after the commute (post1), two hours after the commute (post2) and three hours after the commute (post3), comparing with the MDA levels immediately before the start of commute (pre) are analyzed (Figure 4).

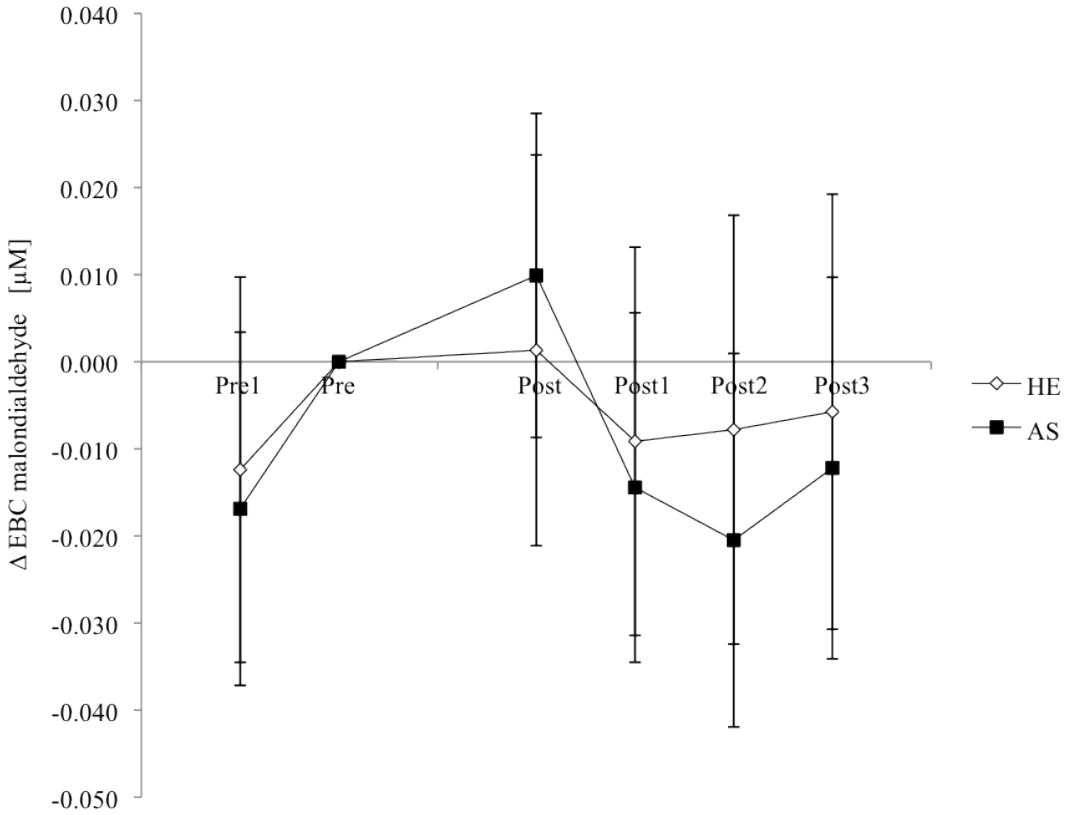


Figure 4. Changes (Δ) in malondialdehyde (MDA) in exhaled breath condensate in effects of exposure to 2-hour commuting. The figure shows the changes (mean and 95% confidence intervals) for malondialdehyde (MDA) in exhaled breath condensate one day before the commute (Pre1), immediately after the commute (Post), one hour after the

commute (Post1), two hours after the commute (Post2) and three hours after the commute (Post3).

The result shown in figure 4 presents an increase immediately after the commute for asthmatics subjects (Post, AS, 0.010, (0.009-0.029) μM). While for healthy subjects, the increase is quite small (Post, HE, 0.001, (-0.021-0.023) μM). Compared with the MDA levels immediately prior to the start of the commute, the changes are negative for MDA levels one day before the commute (Pre1, HE: -0.012, (-0.034-0.010) μM; AS: -0.017, (-0.037-0.003) μM), one hour after the commute (Post1, HE: -0.009, (-0.031-0.013) μM; AS: -0.014, (-0.034-0.006) μM), two hours after the commute (Post2, HE: -0.008, (-0.033-0.017) μM; AS: -0.020, (-0.041-0.01) μM) and three hours after the commute (Post3, HE: -0.006, (-0.031-0.019) μM; AS: -0.012, (-0.034-0.010) μM) for both healthy subjects and asthmatic subjects. The results indicate that the MDA levels in EBC sampled one to three hours after the commute dropped below the levels immediately prior to the start of the commute for both healthy and asthmatic subjects. These changes are all nonsignificant at the 0.05 significance level.

To study the changes of MDA values after finishing the exposure of 2-hour commuting, intraindividual changes in MDA levels between immediately following the commute (Post) and 1-hour intervals for 3 hours following the commute (Post1, Post2, Post3) are analyzed (Figure 5).

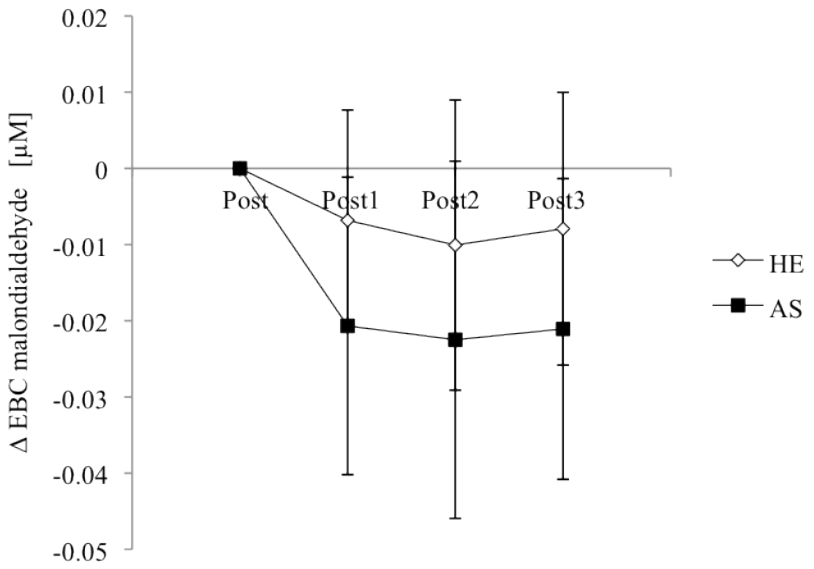


Figure 5. Changes (Δ) in malondialdehyde (MDA) in exhaled breath condensate after exposure of the 2-hour commuting. The figure shows the changes (mean and 95% confidence intervals) for malondialdehyde (MDA) in exhaled breath condensate one hour after the commute (post1), two hours after the commute (post2) and three hours after the commute (post3) compared with the MDA levels immediately after the commute (post).

For healthy subjects, the result presents nonsignificant decreases of MDA levels in EBC sampled one hour after the commute (-0.007, (-0.021-0.007) μM), two hour after the commute (-0.010, (-0.029-0.009) μM) and three hour after the commute (-0.008, (-0.026-0.010) μM). While for asthmatic subjects, the decreases of MDA values for one hour after the commute (-0.021, (-0.041--0.001) μM, p=0.0420) and two hour after the commute (-0.022, (-0.045-0.001) μM, p=0.0173) are significant at the 0.05 significance level.

Associations between air pollutants and MDA levels

Table 6 presents the associations between in-vehicle pollutants and changes of MDA in EBC levels examined by using mixed effect regression models. Several statistically significant associations were seen (Table 6).

Table 6. Malondialdehyde and exposure to in-vehicle air pollutants

Model	LMDA_D_i	Coefficient	SE	P value
Model 1-4: 2-h commute maximum BC ($\mu g/m^3$)	LMDA_D_1	0.0066	0.0047	0.1775
	LMDA_D_2	0.0089	0.0047	0.0753**
	LMDA_D_3	0.0034	0.0054	0.5363
	LMDA_D_4	-0.0007	0.0056	0.8932
Model 5-8: 2-h commute maximum PAH ($\mu g/m^3$)	LMDA_D_1	0.0010	0.0003	0.0065*
	LMDA_D_2	0.0010	0.0003	0.0044*
	LMDA_D_3	0.0011	0.0004	0.0052*
	LMDA_D_4	0.0006	0.0004	0.1219

* Significant at the 0.05 level ** Not significant, but suggestive

Model 1-4 include 2-h commute maximum BC concentration as the independent variable and LMDA_ D_i (log (MDA_D_i: changes in MDA levels between pre and post exposure); D_1 refers to changes between Pre and Post; D_2 refers to changes between Pre and Post1; D_3 refers to changes between Pre and Post2; D_4 refers to changes between Pre and Post3) as the dependent variable. Model 5-8 include 2-h commute maximum PAH concentration as the independent variable instead.

When BC is entered in the model, there is no significant association between BC and changes of MDA levels at the 0.05 significance level. But it shows the effect of BC on the changes in MDA levels between one hour after the commute (Post1) and immediately prior to the start of the commute (Pre) is suggestive (p=0.0753). However, when PAH is entered in the model, several significant associations were seen at the 0.05 significance level. It's shown that the maximum PAH concentrations during the 2-hour

commute are significantly associated with the changes in MDA levels between immediately prior to the commute and 1) immediately following the commute ($p=0.0065$), 2) one hour after the commute ($p=0.0044$) and 3) two-hour after the commute ($p=0.0052$). Moreover, the coefficients are all positive, indicating a positive trend that the changes of MDA levels increase with higher concentration of PAH.

DISCUSSION

I found that subjects have increased levels of MDA in EBC immediately after exposure to the 2-hour commuting. From one hour after the commute, MDA levels decreased to values similar as the MDA levels one day before the commute. These changes are more significant for asthmatic subjects.

Explanation of increased MDA levels

The recent studies that specifically on traffic-related air pollution have largely focused on asthma and related phenomena, with some investigations of allergy. Traffic-related air pollution is a complex mixture of air toxicants including particulate matter, black carbon, and byproducts of incomplete fuel combustion such as PAH, which is a highly reactive organic pollutant that induces oxidative stress in the lung. Oxidative stress in the lungs causes an influx of inflammatory cells to the lung with the subsequent generation. Large quantities of free radicals and reactive oxygen species (ROSs) are released in the lining fluids after exposure.[31]

Since the commonly cited mechanism by which exposure to traffic-related pollutants adversely affect health is oxidative stress, it is logical to measure a biomarker

of oxidative stress injury in the lung. A number of experimental studies have measured biomarker of oxidative stress and inflammation after exposure to traffic-related air pollution. Products of lipid peroxidation are promising markers of oxidative stress in EBC. MDA is one of the main products of the lipid peroxidation in EBC. Further support for MDA in EBC as a suitable biomarker of effect of exposure to traffic-related air pollution comes from a panel study in Mexico City in children with asthma.[28] In agreement with our results, the results in this study show that MDA levels in EBC are increased in relation to increased exposure to traffic-related pollutants. There is a 1.12 nmol increase in MDA associated with increased PM2.5 and a 1.16 nmol increase in MDA associated with increased ozone.[28] Similarly, in my study, I found several significant positive associations between changes of MDA levels with PAH concentrations. PAH has been reported to induce oxidative stress and lipid peroxidation, especially in human lung cells in several studies[32, 33]. The significant association between PAH concentration and the changes in MDA levels between immediately prior to the start of commute and immediately following the commute in my study present a plausible evidence for the effect of in-vehicle air pollutant on oxidative stress. However, there are several confounders that could threat the validity of this inference, including PAH concentrations measured during pre-commuting periods with the continuous monitors, ambient temperature and relative humidity.

Changes of MDA levels between pre and post-exposure

Further more, in my study, the changes of MDA levels associated to effect of the commute is firstly analyzed by comparing the differences of MDA levels between

immediately previous to the start of the commute (Pre) and immediately following the commute (Post). The changes indicate increases of MDA levels after the exposure although they are not significant for either healthy subjects (0.001, (-0.021-0.023) μM) or asthmatic subjects (0.010, (0.009-0.029) μM). Moreover, compared with the changes for healthy subjects, the changes are much bigger for asthmatic subjects. To further study the effect of commute on MDA levels, I compared the MDA levels immediately after the commute (Post) with one hour after the commute (Post1), two hours after the commute (Post2) and three hours after the commute (Post3). The result shows that, when stopping the exposure, the MDA levels decreased fast during the first hour and then maintained on quite consistent levels, which are similar as the MDA levels one day before the commute. The changes are significant for asthmatic subjects only. Thus, if assuming the MDA levels at these 4 time points (Pre, Post1, Post2 and Post3) are the baselines, it is plausible to get the conclusion that the MDA levels for asthmatics increased significantly due to their 2-hour commute. It's not known that why the MDA levels immediately prior to the commute (Pre1) increased compare with one day before the commute (Pre). The reasons of increased MDA levels not related to the commute in our study may include early getting up. Since the commute is in the morning, the subjects have to wake up early to get prepared at around 6 am. The MDA levels increased to indicate their uncomfortable feeling. Also, it is possible that the MDA levels increased during the driving from the subjects' home to our lab prior to the start of the sampling and commute. In addition, there might be a daily trend of MDA levels that it is higher in the early morning and then dropped to normal levels after several hours. Till now there is no specific study to show MDA in EBC daily changes. But in Barregard's study of exposure to wood smoke, which

measured MDA in EBC as the biomarker of oxidative stress as well, there is a decreasing trend of MDA levels in the morning for control group.[34] If this decreasing trend is applicable for our subjects, the increases of MDA levels during the 2-hour commute are more plausible. To get more plausible conclusion, control groups such as clinical visit to our lab without doing 2-hour commute for each subject should be added in the further study.

Different changes of MDA levels between healthy subjects and asthmatic subjects

What's more impressive result from my study is that, the changes of MDA levels is much more significant for asthmatic subjects than for healthy subject. One plausible explanation for this is that the lung has a substantial antioxidant defense capacity varies with the health status of our subjects[35]. Asthmatic subjects may be more susceptible to the effects of traffic-related air pollution during the commute since the oxidant stimulus exceeds the lung's antioxidant defenses. Thus ROS are available to cause oxidative stress injury and increase MDA levels for asthmatic subjects. While for healthy subjects, their antioxidant defenses may overwhelm part of the oxidative stress injury during the commute, resulting in nonsignificant changes of MDA levels. This might be a reason for that most studies on biomarkers of oxidative stress limited their subjects to asthmatics.[36, 22] Any other factor that would have to vary closely with air pollution exposure and affect MDA levels could be confounders in the current study.

Differences of MDA levels between healthy subjects and asthmatic subjects

At each time point, I found MDA levels are higher for asthmatic subjects than healthy subjects. However, I didn't find significant differences of MDA levels between healthy subjects and asthmatic subjects except for the difference of MDA levels immediately after the commute, which is suggestive through not significant. Some studies have shown that, in asthmatic patients, EBC-MDA levels were higher than healthy subjects ($p<0.001$).[37] This discrepancy may be ascribed to the different populations studied and to the different methods of measurements used.

In my experience, MDA measurement in EBC using HPLC was quite reproducible. I randomly selected 12 EBC samples and did the duplication after finishing all the sample analysis (Figure 6). Most of the MDA levels increased during duplication. The repeatability is quite plausible because some studies have indicated that MDA levels would increase significantly after 6 months of storage.[38] Since the long duration of my lab work, some of the selected samples have been stored again after their first analysis for more than 6 months. Thus, it is reasonable to get higher levels of MDA for duplication. For recommendation of further studies, the storage time of EBC samples should be maintained within 6 months and be measured timely.

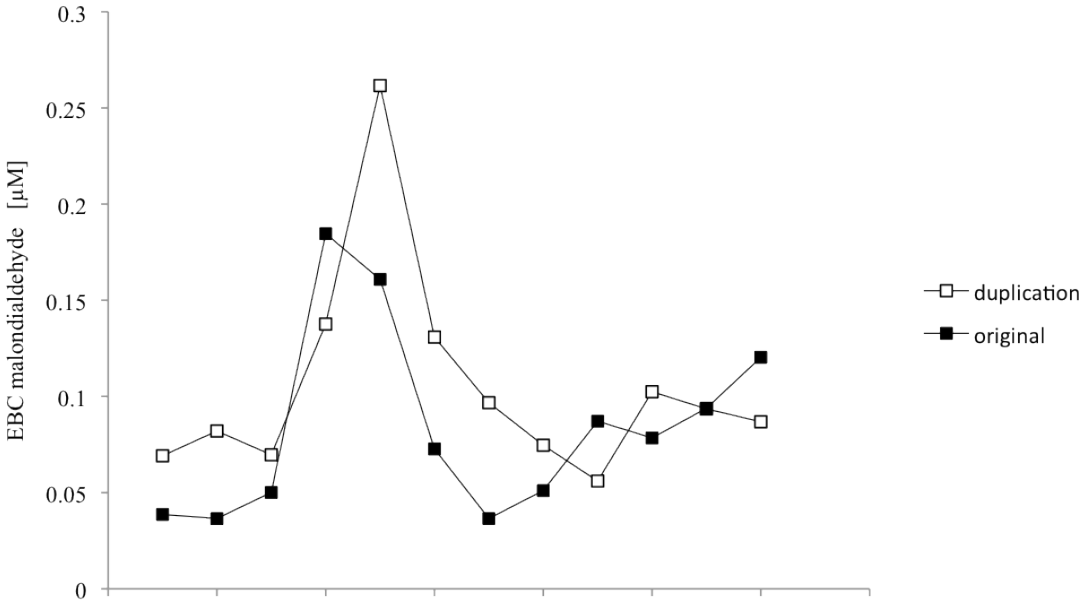


Figure 6. Duplication of randomly selected EBC-MDA samples.

Methodology limitation

MDA is the major product of lipid peroxidation and has been found in EBC. EBC can closely reflect the composition of epithelial lining fluid because there is great surface area from which generation of aerosols may occur.[39] Moreover, there has been more interest in measure compounds in EBC because of the easy collection of samples. However, the method used to collect EBC is still under development. There are methodological difficulties including the problem of oral contamination of the exhaled air. Salivary contamination may influence the MDA levels in EBC, so it's important to minimize salivary contamination. We direct our subjects by following the proposal mentioned before to reduce contamination from constituents of saliva. Meanwhile, all the samples are assessed for salivary contamination by testing polyatomic ion phosphate (PO_4^{3-}) concentrations using ion chromatography (IC). High concentration of PO_4^{3-} in

EBC would suggest contamination of saliva. For all of my samples, the concentrations of PO_4^{3-} are all low, indicating no contamination from saliva.

Another limitation of EBC method is the high variability in measurements. The major component of EBC is condensed water vapor and this may lead to variable dilution of EBC constituents. This is one of the factors contributing to the variability in measurements of MDA levels in my study. The sample dilution issue is not necessary to worry in the analysis of interindividual changes of MDA levels because the compared samples were from the same subject and their sample dilution are similar. However, when compare the MDA levels between healthy subjects and asthmatic subjects, sample dilution may decrease the power of my conclusion. To control for dilution and decrease variability in measurements, we collected EBC over a specified duration of collection at defined temperature. It is reported in one study that MDA levels in samples collected at 10 and 20 min were similar.[40] Whereas other authors showed decreased MDA levels with prolonged sampling time.[41] We collected EBC samples using a standardized EBC collection time of 10 min. For the temperature of collection, it remains unclear for MDA what the optimum temperature is. Most investigators believe that the yield of mediators is higher in colder collection temperatures. Our RTube sleeves used for EBC collection were chilled to -80 °C consistently. The collection methods we employed could have resulted in type II error because of high variability between subjects; however, we have shown consistent results within subjects using a standardized EBC collection time of 10 min and temperature of -80 °C.

There has not been a robust methodology for EBC analysis established yet. The lack of standardization of EBC analysis is currently the primary limitation of this

technique and is the major explanation of the variability of the results reported in different studies. Current methodological limitations of EBC analysis that need to be addressed in future studies include possible saliva and sputum contamination; influence of temperature and duration of sample collection and storage issues.[42]

CONCLUSION

In conclusion, I demonstrated that exposure to traffic related air pollution affects the airways inflammation, as shown by the changes in MDA in exhaled breath condensate. The changes of MDA levels for asthmatic subjects are more significant than healthy subjects. . From one hour after the commute, MDA levels decreased to constant values similar as the MDA levels one day before the commute. Moreover, there are significant associations between the in-vehicle pollutants (especially PAHs) and the changes of MDA levels between pre and post-exposure. My study also proved the usefulness of EBC-MDA as a biomarker of effect of exposure to traffic-related air pollutants. The measurements provide insight into important aspects of both PM exposure assessment and epidemiology. Results will further the understanding of the respective influences of inflammatory mechanisms of PM mediated pulmonary responses. Moreover, results provide a question to the society that whether urban commuters should be viewed as a susceptible sub-population given their enhanced exposures to traffic-related air pollutants.

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