MICRORNAS IN EXTRACELLULAR VESICLES AS NOVEL BIOMARKERS IN ENVIRONMENTAL EPIDEMIOLOGY

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ABSTRACT

Despite important achievements of Environmental Epidemiology in identifying major environmental health problems in recent years, little is known about the underlying biological mechanisms that link environmental exposures to certain outcomes. Recent discovery of extracellular vesicles (EVs) as a novel mechanism of cellular communication has opened new avenues for non-invasive access to subclinical molecular signals, which might help exposing such mechanisms. In this work, we evaluate the potential of microRNAs, molecular signals that are contained in EVs circulating in the blood, as biomarkers in two very important environmental health problems: (a) the causal association between air pollution and cardiovascular disease and (b) the association between prenatal lead exposure and impaired fetal growth.

For this work, we used cutting-edge and high-throughput methods to isolate EVs from the blood of study participants, and profile their content in microRNAs (evmiRNAs). We further incorporated a broad array of sophisticated statistical methods to (a) determine the association of ambient particulate matter of <2.5μm diameter (PM$_{2.5}$) and evmiRNAs (Chapter 1), (b) determine the association between prenatal lead exposure and fetal growth (Chapter 2), and (c) evaluate the potential of evmiRNAs as early biomarkers of fetal growth impairment (Chapter 3).

The findings from Chapter 1 showed positive associations between long-term exposure to PM$_{2.5}$ and levels of evmiRNAs in the blood circulation of older individuals, several of which were found to be enriched in biological pathways related to cardiovascular disease. Results from Chapter 2 showed a negative association between prenatal exposure to lead and birthweight-for-gestational age (BWGA), and revealed that the magnitude of the association is
much bigger in the lower percentiles of the BWGA distribution. Finally, the findings from Chapter 3 suggested that levels of evmiRNAs provide a very promising opportunity for the development of minimally-invasive biomarkers to detect and monitor impaired fetal growth early in pregnancy.

This work demonstrates the tremendous potential of evmiRNAs in the field of Environmental Epidemiology. EVs and their content provide an unprecedented, yet easily accessible source of molecular signals that can help us better understand the biological mechanisms involved in linking environmental exposures to certain outcomes.
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INTRODUCTION
Recent advances in biological sciences revealed that Extracellular Vesicles (EVs), previously thought to be cellular debris, provide a novel mechanism of communication between cells. EVs are nano-sized (0.05-1 μm) membrane vesicles that are released from all types of cells in the extracellular space, and that can be detected in a wide spectrum of biofluids such as the blood, urine, saliva, and breast milk (Yanez-Mo et al. 2015). Upon their release and in biofluids, EVs can travel to distant cell(s) or tissue(s) and internalized either by fusion with the cellular membrane, endocytosis, or by a ligand-receptor interaction (Katzmann et al. 2002; Simons et al. 2009; Thery et al. 2009). Following their uptake by the recipient cell(s), EVs can then deliver their content (e.g., proteins, RNA, and DNA molecules) in the intracellular space (Skog et al. 2008). microRNAs (miRNAs) are short non-coding RNA molecules of 22-25 nucleotides length that are abundantly present in EVs (evmiRNAs). It has been shown that evmiRNAs that are transferred from cell-to-cell become functional once internalized by the recipient cell(s) (Mittelbrunn et al. 2011). The role of miRNAs has been shown to be catalytic in post-transcriptional gene regulation, either by directly targeting and degrading messenger RNA (mRNA) molecules with complementary sequences, or by inhibiting the translational machinery of the cell (Bartel 2009). Considering that mRNA molecules provide the only template that codes to proteins, miRNAs can be described as a mechanism that post-transcriptionally modulates the cell’s gene expression. It is estimated that miRNAs can regulate ~50% of the human gene expression (Krol et al. 2010). Therefore, circulating EVs in blood represent a unique opportunity to minimally-invasively harvest cellular signals from tissues that come in contact with easily accessible biofluids (e.g., blood, urine, salive, etc.). Being able to decode these signals might provide us with valuable information during different stages of disease, spanning from early diagnosis to clinical intervention monitoring. Despite the growing interest in EVs in the field of biomedical sciences, their potential as biomarkers in the fields of environmental and public health has not been evaluated.
An ideal setting to evaluate the potential of evmiRNAs as biomarkers is the example of the casual association we are consistently seeing between air pollution and Cardiovascular Disease (CVD). In the United States (U.S.) alone, 31% (2.5 million) of all deaths are attributed to CVD, and is expected to considerably increase by 2030 (Organisation 2014). Health care expenses attributed to CVD were estimated to be ~580 billion dollars for 2012, and is expected to increase to ~1.2 trillion dollars, following the increase in CVD prevalence in the upcoming years (Mozaffarian et al. 2015). Ambient air pollution is one of the most considerable risk factors for the development of CVD. It has been estimated that air pollution is linked to nearly three million CVD related deaths annually (Organisation 2014). Particulate matter with an aerodynamic diameter of 2.5 μm or less (PM_{2.5}) can penetrate deep into the lung and locally trigger oxidative stress and inflammatory responses. Epidemiological studies have consistently shown a substantial increase (3-76%) in cardiovascular mortality in response to particulate matter exposures (Brook et al. 2010). Few biological mechanisms have been proposed to play a role in mediating the effects of PM_{2.5} from the lung to the cardiovascular system, such as systemic inflammation. Several inflammatory markers, including C-reactive protein (CRP) (Hoffmann et al. 2009), interleukin (IL)-6 (Ruckerl et al. 2007), fibrinogen (Ghio et al. 2003), and platelet activation (Rudez et al. 2009) were found to be elevated in the blood in response to PM exposures. However, the mechanisms through which PM_{2.5} inhalation leads to CVD remain to be fully understood. In a systematic review, Vrijens et al. summarized data from human studies showing that miRNA levels change in response to air pollution exposures, primarily in exposed tissues (Vrijens et al. 2015). Whether the levels of evmiRNAs in blood circulation reflect environmental exposures, including ambient PM_{2.5} is still largely undetermined. Therefore, elucidating the subclinical biological changes that precede the onset of CVD, and are associated with air pollution, will help us to identify susceptible populations and inform new preventive policies to promote public health.
Another major public health problem where EVs can significantly contribute is the example of prenatal exposure to environmental toxins and fetal growth. It is well established that impaired fetal growth, including both fetal growth restriction and overgrowth, is a prevalent cause of perinatal deaths in high risk pregnancies (Bernstein et al. 2000) and is also associated with chronic and metabolic disorders for the infant later in life (Barker et al. 2002; Oken et al. 2003). The macro-economic and social impact of impaired fetal growth on a country’s human capita is tremendous (Barker 2006; Reilly et al. 2003). The placenta is a critical organ for fetal growth that facilitates several processes to guarantee normal fetal development and healthy course of pregnancy. Not only does placenta enable the transfer of oxygen and nutrients from the mother to the fetus, but it is also involved in several other biological processes such as hormonal signaling and immunomodulation (Thornburg et al. 2015). Therefore, it is easily understood that placental dysfunction can have a detrimental effect on fetal development. Lead is a well-known toxic metal that has been extensively studied and linked to multiple birth outcomes including poor fetal growth and spontaneous abortion (Hertz-Picciotto 2000). Few studies suggested that lead can reach the placenta and the fetus via the maternal blood circulation, and affect fetal development by competing with essential nutrients such as calcium and zinc or by triggering oxidative stress conditions which lead to impaired fetal development (Needleman et al. 1984). However, the exact underlying mechanisms that explain this association, and that are involved in feto-maternal communication, remain largely unknown. Recent evidence has demonstrated that EVs of placental origin, detected in the maternal blood circulation, can target and reprogram other cells in maternal and fetal tissues by transferring proteins and non-coding RNAs (e.g., miRNAs) (Chen et al. 2012). Most of the studies have focused on the placenta specific miRNA clusters on chromosomes 14 (C14MC) (Bentwich et al. 2005) and 19 (C19MC) (Mouillet et al. 2010) that are highly expressed in the placenta, and that are usually detected in EVs circulating in maternal blood. Several miRNAs from these clusters are implicated in regulating genes critical to placental development and immunomodulation.
during gestation (Mouillet et al. 2015). Whether their levels in circulating EVs in maternal blood can predict fetal growth early in pregnancy and provide insights on the underlying mechanisms linking the impact of environmental exposures (e.g., lead) on fetal growth, remains to be elucidated.

**Dissertation goal**

The goal of this dissertation was to determine the potential of miRNAs encapsulated in extracellular vesicles as easily-accessible biomarkers to help us better understand in real time key underlying mechanisms that are involved different stages of disease, as well as to identify high risk individuals in relation to certain environmental exposures. To address this goal, we leveraged the existence of an ongoing prospective cohort study of older individuals in the Great Boston area; a high risk subpopulation for the development of CVD in response to air pollution (Chapter 1). The dissertation did not examine any associations between air pollution and CVD (hence shown in solid line in Figure 0.1). We further leveraged another ongoing prospective birth cohort of mother-infant pairs in Mexico City who might be at higher risk for the effects of lead on fetal growth (Chapters 2 and 3). An overview of the specific dissertation aims (dotted lines) is shown below:
Figure 0.1: Dissertation overview. Chapter 1 examines the association between air pollution and levels of miRNAs in extracellular vesicles, and explores their biological relevance in the development of cardiovascular disease. Chapter 2 determines the association between prenatal lead exposure and fetal growth, and Chapter 3 evaluates the potential of miRNAs in extracellular vesicles as biomarkers of fetal growth early in pregnancy.
REFERENCES


"Biological Properties of Extracellular Vesicles and Their Physiological Functions."
CHAPTER 1

Ambient Particulate Matter and MicroRNAs in Extracellular Vesicles: A Pilot Study of Older Individuals

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ABSTRACT

Background

Air pollution from particulate matter (PM) has been linked to cardiovascular morbidity and mortality; however the underlying biological mechanisms remain to be uncovered. Gene regulation by microRNAs (miRNAs) that are transferred between cells by extracellular vesicles (EVs) may play an important role in PM-induced cardiovascular risk. This study sought to determine if ambient PM$_{2.5}$ levels are associated with expression of EV-encapsulated miRNAs (evmiRNAs), and to investigate the participation of such evmiRNAs in pathways related to cardiovascular disease (CVD).

Methods

We estimated the short- (1-day), intermediate- (1-week and 1-month) and long-term (3-month, 6-month, and 1-year) moving averages of ambient PM$_{2.5}$ levels at participants’ addresses using a validated hybrid spatio-temporal land-use regression model. We collected 42 serum samples from 22 randomly selected participants in the Normative Aging Study cohort and screened for 800 miRNAs using the NanoString nCounter® platform. Mixed effects regression models, adjusted for potential confounders were used to assess the association between ambient PM$_{2.5}$ levels and evmiRNAs. All p-values were adjusted for multiple comparisons. In-silico Ingenuity Pathway Analysis (IPA) was performed to identify biological pathways that are regulated by PM-associated evmiRNAs.

Results

We found a significant association between long-term ambient PM$_{2.5}$ exposures and levels of multiple evmiRNAs circulating in serum. In the 6-month window, ambient PM$_{2.5}$ exposures were associated with increased levels of miR-126-3p (0.74 ± 0.21; p=0.02), miR-19b-3p (0.52 ± 0.15; p=0.02), miR-93-5p (0.78 ± 0.22; p=0.02), miR-223-3p (0.74 ± 0.22; p=0.02), and miR-142-3p
(0.81 ± 0.21; p=0.03). Similarly, in the 1-year window, ambient PM$_{2.5}$ levels were associated with increased levels of miR-23a-3p (0.83 ± 0.23; p=0.02), miR-150-5p (0.90 ± 0.24; p=0.02), miR-15a-5p (0.70 ± 0.21; p=0.02), miR-191-5p (1.20 ± 0.35; p=0.02), and let-7a-5p (1.42 ± 0.39; p=0.02). In silico pathway analysis on PM$_{2.5}$-associated evmiRNAs identified several key CVD-related pathways including oxidative stress, inflammation, and atherosclerosis.

**Conclusions**

We found an association between long-term ambient PM$_{2.5}$ levels and increased levels of evmiRNAs circulating in serum. Further observational studies are warranted to confirm and extend these important findings in larger and more diverse populations, and experimental studies are needed to elucidate the exact roles of evmiRNAs in PM-induced CVD.

**Keywords**: Air Pollution; Particulate Matter; Cardiovascular Disease; Extracellular Vesicles; microRNAs;
BACKGROUND

Cardiovascular disease (CVD) is a leading cause of mortality and morbidity worldwide. In the United States (U.S.) alone, 31% (2.5 million) of all deaths are attributed to CVD, and the proportion is expected to increase to ~40% by 2030. The total health care cost of CVD was estimated to be ~580 billion dollars for the year 2012, but is projected to peak at ~1.2 trillion dollars by 2030 (Mozaffarian et al. 2015). Among the risk factors for CVD, air pollution confers considerable risk. In fact, ambient air pollution has been estimated to account for ~3.7 million global deaths annually, and 80% of those deaths have been attributed to CVD (WHO 2014). Associations of short- and long-term exposures to air particulate matter (PM) with increased CVD mortality and morbidity are, indeed, well documented (Brook et al. 2010; Pope et al. 2004). Despite significant past and recent efforts to reduce emissions, PM$_{2.5}$ is still associated with CVD, even at the current levels (Madrigano et al. 2013). Elucidating the subclinical biological changes that precede the onset of CVD, and are associated with air pollution, will help us to identify susceptible populations and inform new preventive policies to promote public health.

Particulate matter with an aerodynamic diameter of 2.5 μm or less (PM$_{2.5}$) can penetrate deep into the lung, deposit in the alveolar area, and locally trigger oxidative stress and inflammatory responses. Epidemiological studies have consistently shown a substantial increase (3-76%) in cardiovascular mortality in response to particulate matter exposures (Brook et al. 2010). Experimental studies have revealed that compromised vascular and cardiac functions such as systemic inflammation (Thompson et al. 2010), oxidative stress (Chuang et al. 2007), and heart rate variability (Chahine et al. 2007) due to prolonged exposure to PM$_{2.5}$, precede serious CVD complications that often lead to death. Few biological mechanisms have been proposed to play a role in mediating the effects of PM$_{2.5}$ from the lung to the cardiovascular system. One such mechanism is systemic inflammation, as several inflammatory markers, including C-reactive protein (CRP) (Hoffmann et al. 2009), interleukin (IL)-6 (Ruckerl et
al. 2007), fibrinogen (Ghio et al. 2003), and platelet activation (Rudez et al. 2009) were found to be elevated in the blood in response to PM exposures. However, the mechanisms through which PM$_{2.5}$ inhalation leads to CVD remain to be fully understood.

Extracellular vesicles (EVs) are small (40-1000 nm in diameter) double-lipid membrane vesicles (Mathivanan et al. 2010) that play an important role in the cell-to-cell communication process (Yanez-Mo et al. 2015). EVs encapsulate and transfer biologically active molecules such as proteins and RNA molecules. Once EVs are internalized by the recipient cell(s), their cargo is released to the cytosol and could become functional (Valadi et al. 2007). microRNAs (miRNAs), small non-coding RNAs (~22 nucleotides long) (Bartel 2009) are abundantly present in EVs (Mathivanan et al. 2010). Because of their ability to degrade and/or suppress the translation of multiple mRNA molecules, miRNAs can post-transcriptionally regulate gene expression in cells (Guo et al. 2010). In a systematic review, Vrijens et al. summarized data from recent human studies showing that miRNA levels change in response to air pollution exposures (Vrijens et al. 2015). Izzoti et al., reviewed evidence suggesting that dysregulation of miRNA expression levels in response to persistent environmental exposures occurs primarily due to their interaction with components of the miRNA machinery such as the Drosha/DGCR8 processing complex, DICER and RNA-induced silencing complex (RISC) (Izzotti et al. 2014). However, whether the levels of evmiRNAs in blood circulation are sensitive to environmental exposures, including ambient PM$_{2.5}$ is still largely undetermined.

The objective of our study was to determine whether short-, intermediate-, and long-term exposures to ambient PM$_{2.5}$ are associated with the levels of evmiRNAs detected in the blood of older individuals. We screened for 800 miRNAs in 42 serum samples of 22 randomly selected participants from the ongoing prospective Normative Aging Study (NAS). Additionally, we conducted pathway analysis to explore whether PM$_{2.5}$-associated evmiRNAs regulate in CVD-
related biological pathways. The findings of this study offer new avenues to explore the link between air pollution and CVD.

METHODS

Sample selection
The U.S. Department of Veterans Affairs (VA) Normative Aging Study is an ongoing longitudinal study established in 1963, which enrolled 2,135 male volunteers, aged 21 to 80 years of age at enrollment from the Greater Boston area. All participants were free of any known chronic medical conditions at baseline. Further details of the study have been described previously (Bell et al. 1966). Participants were invited to undergo comprehensive medical examinations every 3 to 5 years. By 2000, when satellite air pollution data became available, 749 of the original participants were still attending regular examinations. In this pilot study, we randomly selected 22 participants with available serum samples between the years 2000-2008. One serum sample from each participant at first visit (n=22), and two additional samples for 10 out of 22 participants, from the following two visits (n=20), were selected. In total, we analyzed 42 serum samples. The NAS was approved by the Institutional Review Boards of all participating institutions (#14027-102, reviewed on March 20, 2012). All participants provided written informed consent in accordance with the Declaration of Helsinki of ethical principles for medical research.

Exposure assessment
A validated spatio-temporal land-use regression model was used to estimate the ambient moving-average PM$_{2.5}$ levels at each participant’s home address for all time windows (1 day, 1 week, 1 month, 3 months, 6 months, and 1 year) preceding each blood withdrawal. We used our
recently developed hybrid approach (Kloog et al. 2012), which combines satellite data on aerosol optical density (AOD) with land-use variables, together with weather data and PM$_{2.5}$ point- and area-source emissions from 78 monitoring stations across the study area from the U.S. Environmental Protection Agency (EPA) – National Emission Inventory ((USEPA) 2010). We validated and successfully used these estimates in several recent investigations (Chiu et al. 2014; Kloog et al. 2015). In brief, this model uniquely integrates satellite AOD data, which are a measure of light scattering due to the presence of aerosols in the column of air spanning from Earth’s surface to the satellite. To estimate the levels of PM$_{2.5}$ near the surface, we fitted a hybrid model using AOD and classic land-use regression data (i.e., elevation, distance to major roads, % open space, point emissions, and area emissions) together with several other meteorological variables such as temperature, wind speed, relative humidity, visibility, and height of the planetary boundary layer (PBL). Additional information on the development of this model is available elsewhere (Kloog et al. 2011). The model includes interaction terms with the PBL height to help capture differences in the fraction of particles that are near the ground and random slopes for each day to capture day-to-day changes in particle size and color. We also estimated daily PM$_{2.5}$ concentration levels for all grid cells in the study domain for cells/days when AOD data were unavailable (e.g., cells covered by clouds) using grid-cell specific regressions against nearby monitors, land-use terms, and spatial smoothing. A final stage examined the difference of each daily PM$_{2.5}$ observation on a 10x10-km grid cell mean. Differences were regressed against land-use terms within 100 m of the monitor, meteorology, and their interactions. This allows us to resolve the address-specific exposure. Ten-fold cross-validation showed very good model predictions with an out of sample $R^2$ of 0.85 for daily PM$_{2.5}$ measures.
Blood collection, EVs isolation, and miRNA extraction

Peripheral blood was collected at each visit in EDTA tubes and centrifuged at 1500 x g for 15 min to separate the serum fraction following standard operating procedure. Aliquots of cell-free serum were stored immediately at -80°C and thawed just before use for this study. For the isolation of EVs and miRNA extraction we used the ultracentrifugation method as first described by Théry et al. (Thery et al. 2006) with some modifications (Bollati et al. 2014). In brief, serum samples (~1.5 mL) were thawed on ice and centrifuged at 1000, 2000, and 3000 x g for 15 min at 4°C, consecutively, to remove any remaining cell debris and large aggregates. Supernatants were then filtered using a 0.8-μm membrane unit (Millipore Corp., Bedford, MA) and further centrifuged using a TLA-110 fixed-angle rotor (Beckman Coulter, Danvers, MA) at 110,000 x g (k factor of 13) for 2 h at 4°C. Isolated EVs were visualized by transmission electron microscopy and immune-gold labeling using antibodies for the CD-63 and CD-81 surface markers as described by Théry et al. (Thery et al. 2006). (Figure S1.1). Finally, miRNAs were extracted from the collected EVs using the miRNeasy Mini Kit (Qiagen, Valencia, CA) according to manufacturer’s instructions, and the RNA eluate was concentrated for downstream analysis using a vacuum concentrator.

miRNA profiling

The Nanostring nCounter® platform was used to screen for expression level of 800 miRNAs. A volume of three microliters (3 μL) for each sample was prepared and analyzed according to the manufacturer’s protocol (NanoString Technologies, Seattle, WA). Briefly, a thermally controlled multiplexed ligation reaction was used to add specific DNA tag sequences on mature miRNAs. Following ligation, the excess tags were removed by affinity and the purified material was hybridized overnight at 65°C with the nCounter® Human (V2) miRNA Expression Assay CodeSet. The nCounter® Prep Station was used to purify the hybridized probes and to attach the purified biotinylated complexes on the streptavidin-coated slides. miRNA counts were
measured in two batches by the nCounter® Digital Analyzer. All samples were analyzed at NanoString’s laboratory (NanoString Technologies, Seattle, WA). The nSolver software (http://www.nanostring.com/products/nSolver) was used to analyze and normalize the raw data using the top 100 most abundant miRNAs in all samples, according to the manufacturer’s instructions. Positive controls were included to normalize for any differences in preparation, hybridization, and processing efficiency. Data were further tested for batch effects, normalized to the starting median serum volume and corrected for background noise using negative controls (internal probes and biological blank). Normalized miRNA counts were used for further analysis.

**Statistical analysis**

Standard descriptive statistics were used to explore the characteristics of the study participants and the levels of evmiRNAs circulating in serum [reported as mean ± standard deviation (SD)]. We log-transformed (log₂) the evmiRNA data to improve normality in the residuals. Univariate analysis was conducted between all PM$_{2.5}$ moving averages (1-day, 1-week, 1-month, 3-month, 6-month, and 1-year) and all detected evmiRNAs. To maximize the completeness of our data set in the multivariate analysis, we included only the evmiRNAs that were detected in >90% of our samples. For our analysis on the association of PM$_{2.5}$ on the levels of evmiRNAs, we adjusted for other covariates such as age, body mass index (BMI), pack-years of smoking, total miRNA counts, and the numbers of red blood cells (RBCs), white blood cells (WBCs), and platelets. Additional covariates such as seasonality and years of education were also evaluated in our preliminary analysis; however, none of them improved the performance of our statistical models or significantly changed our findings; thus, we decided to use a more parsimonious model. The same covariates were used to explore the association between evmiRNAs and coronary heart disease (CHD) history (yes/no) in the study participants, using logistic regression models. To incorporate all data from the repeated measures of each of the detected evmiRNAs
in our study population, the mixed effects models approach with random intercept for each participant was used. The Benjamini and Hochberg (BH) procedure was used to control for multiple comparisons (n=186, 31 miRNAs x 6 time windows) and False Discovery Rate (FDR) (Yoav Benjamini 1995). A two-sided BH FDR of <0.05 was considered significant. Lastly, Spearman’s correlation analysis was conducted to explore the longitudinal correlations of the evmiRNAs in repeatedly collected serum samples. All statistical analyses were conducted using SAS version 9.4 (SAS Institute Inc., Cary, NC).

**miRNA targets and biological network analysis**

We used the Ingenuity Pathway Analysis (IPA) software (Ingenuity Systems®, Redwood City, CA) to identify miRNA putative targets and explore downstream biological networks. Only evmiRNAs that were found to be associated with ambient PM$_{2.5}$ levels were included in this analysis. The miRNA Target Filter tool, which links predicted and experimentally validated mRNA targets to each miRNA from TarBase, miRecords and TargetScan, was used. In the pathway and biological network analysis, we used the following parameters: (a) Confidence level: Experimentally Observed, (b) Species: Human and (c) Biofluids: Blood and Serum/Plasma. Further *in silico* analysis was conducted to explore the biological relevance of PM$_{2.5}$-associated evmiRNAs and signaling pathways related to the cardiovascular system and inflammatory responses, that we selected *a priori*.

**RESULTS**

**Characteristics of study participants**

In this study, all participants (n=22) were non-hispanic white men, with a mean age of 75 ± 6.6 years, BMI of 26.8 ± 2.9 kg/m$^2$, RBC counts of 4.7 ± 0.4 thousands/mm$^3$, WBC counts of 6.5 ± 2.5 thousands/mm$^3$, and platelet counts of 231.9 ± 51.8 thousands/mm$^3$, at the first examination
Sixteen participants (62.8%) were former smokers and six (37.2%) never smoked. Former smokers quitted smoking at least 12 years prior to enrollment and reported a mean of 15.8 ± 15.2 pack-years of smoking. Seven participants (31.8%) had a history of CHD at baseline. The ambient PM$_{2.5}$ levels (mean ± SD) for 1-day, 1-week, 1-month, 3-month, 6-month, and 1-year moving averages were estimated to be 10.76 ± 6.85 μg/m$^3$, 10.48 ± 2.81 μg/m$^3$, 10.32 ± 2.36 μg/m$^3$, 11.05 ± 1.78 μg/m$^3$, 11.14 ± 1.14 μg/m$^3$, and 11.14 ± 0.97 μg/m$^3$, respectively.

**Association of ambient PM$_{2.5}$ levels and evmiRNAs in serum**

We determined the association between ambient PM$_{2.5}$ exposures and levels of evmiRNAs circulating in serum using short- (1-day), intermediate- (1-week and 1-month), and long-term (3-month, 6-month, and 1-year) PM$_{2.5}$ moving averages. The volcano plots depict the univariate association (fold change) per SD increase of PM$_{2.5}$ on all evmiRNAs detected in at least one sample (n=798), at all tested PM$_{2.5}$ moving averages (Figure 1.1; Table S1.2). Of the 798 detected evmiRNAs, 31 were present in >90% of all analyzed serum samples and thus selected for further analysis. In Table 1.1, we show the levels and number of serum samples in which each of the 31 evmiRNAs was detected. Mixed effects regression models, adjusted for age, BMI, pack-years of smoking, total miRNA counts, and counts of RBCs, WBCs, and platelets, revealed a statistically significant long-term (6-month and 1-year) association of ambient PM$_{2.5}$ exposures and levels of evmiRNAs circulating in serum. All results were adjusted for multiple comparisons using FDR correction. Of the 31 tested evmiRNAs, we found that 16 were statistically significantly associated with either the 6-month or 1-year PM$_{2.5}$ moving averages.

We herein show the top-5 evmiRNAs (by p-value) for each PM$_{2.5}$ moving average time window and their fold-change levels in response to every PM$_{2.5}$ SD increment (Table 1.2).

We found the most significant associations between 6-month PM$_{2.5}$ moving average and fold change of miR-126-3p (0.74 ± 0.21; p=0.02), miR-19b-3p (0.52 ± 0.15; p=0.02), miR-93-5p...
(0.78 ± 0.22; p=0.02), miR-223-3p (0.74 ± 0.22; p=0.02), and miR-142-3p (0.81 ± 0.21; p=0.03). Furthermore, for the 1-year PM$_{2.5}$ moving average, miR-23a-3p (0.83 ± 0.23; p=0.02), miR-150-5p (0.90 ± 0.24; p=0.02), miR-15a-5p (0.70 ± 0.21; p=0.02), miR-191-5p (1.20 ± 0.35; p=0.02), and let-7a-5p (1.42 ± 0.39; p=0.02) showed the most significant associations. The complete list of all evmiRNAs and their fold changes in response to short-, intermediate-, and long-term PM$_{2.5}$ moving averages are shown in Table S1.2 (Supplemental Material).

Figure 1.1: Univariate association of ambient PM$_{2.5}$ levels and all measured miRNAs (n=798) in extracellular vesicles (EVs).

PM$_{2.5}$ 1-day, 1-week, 1-month, 3-month, 6-month, and 1-year indicate the ambient PM$_{2.5}$ moving average levels one day, one week, one month, three months, six months, and one year before blood sample collection; -log$_{10}$ (P values) indicates log$_{10}$ transformed p-values of the fold-change association of PM$_{2.5}$ moving average time windows on each of the detected miRNAs in
serum extracellular vesicles; fold change (log$_2$) indicates the log$_2$ transformed fold change of each of the detected miRNAs in serum extracellular vesicles in response to PM$_{2.5}$ moving average time windows.

Table 1.1: Levels of miRNAs in extracellular vesicles detected in >90% of analyzed serum samples (n=42).

<table>
<thead>
<tr>
<th>miRNA</th>
<th>n (obs)$^*$</th>
<th>Mean (SD)$^\dagger$</th>
</tr>
</thead>
<tbody>
<tr>
<td>let-7a-5p</td>
<td>41</td>
<td>6.25 (2.60)</td>
</tr>
<tr>
<td>let-7b-5p</td>
<td>41</td>
<td>5.23 (1.88)</td>
</tr>
<tr>
<td>let-7g-5p</td>
<td>42</td>
<td>8.24 (1.20)</td>
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<td>miR-106b-5p</td>
<td>41</td>
<td>5.16 (1.35)</td>
</tr>
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<td>miR-1246</td>
<td>41</td>
<td>2.55 (3.87)</td>
</tr>
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<td>miR-126-3p</td>
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<td>8.16 (1.41)</td>
</tr>
<tr>
<td>miR-130a-3p</td>
<td>42</td>
<td>5.72 (1.02)</td>
</tr>
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<td>miR-142-3p</td>
<td>42</td>
<td>8.68 (1.36)</td>
</tr>
<tr>
<td>miR-144-3p</td>
<td>42</td>
<td>8.83 (1.25)</td>
</tr>
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<td>miR-146a-5p</td>
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<td>5.15 (2.12)</td>
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<td>7.85 (1.86)</td>
</tr>
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<td>miR-15a-5p</td>
<td>42</td>
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<td>miR-15b-5p</td>
<td>42</td>
<td>7.27 (1.24)</td>
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<tr>
<td>miR-16-5p</td>
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<td>8.24 (1.57)</td>
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<td>6.27 (1.56)</td>
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<tr>
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<td>5.16 (2.07)</td>
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<td>miR-223-3p</td>
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<td>9.70 (1.43)</td>
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<td>miR-4454</td>
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<td>miR-451a</td>
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<td>miR-505-3p</td>
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<td>miR-720</td>
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<td>6.18 (1.41)</td>
</tr>
<tr>
<td>miR-93-5p</td>
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<td>6.30 (1.81)</td>
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</table>

$^*$Number of serum samples in which each miRNA was detected  
$^\dagger$Expressed as log$_2$
<table>
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<tr>
<th>Exposure window</th>
<th>miRNA</th>
<th>Fold change</th>
<th>SE</th>
<th>FDR adjusted P value</th>
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<td>0.17</td>
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</tr>
<tr>
<td></td>
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<td>0.14</td>
<td>0.16</td>
</tr>
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<td>miR-342-3p</td>
<td>-0.44</td>
<td>0.22</td>
<td>0.20</td>
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<tr>
<td></td>
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<td>-0.56</td>
<td>0.29</td>
<td>0.23</td>
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<td></td>
<td>miR-451a</td>
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<td>0.16</td>
<td>0.30</td>
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<td>1-week</td>
<td>miR-451a</td>
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<td>0.16</td>
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<td>0.14</td>
<td>0.37</td>
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<td>miR-720</td>
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<td>miR-4454</td>
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<td>0.44</td>
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</tr>
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<td>miR-142-3p</td>
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<td>miR-126-3p</td>
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<td>0.21</td>
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<td>0.22</td>
<td>0.02</td>
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<td></td>
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<td></td>
<td>miR-142-3p</td>
<td>0.81</td>
<td>0.21</td>
<td>0.03</td>
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<td>0.02</td>
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<tr>
<td></td>
<td>let-7a-5p</td>
<td>1.42</td>
<td>0.39</td>
<td>0.02</td>
</tr>
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</table>
(Table 1.2 Continued)

*Estimated fold change \((\log_2)\) of the levels of evmiRNAs per standard deviation (SD) increase in ambient PM\(_{2.5}\) levels for every time window; all models were adjusted for age, body mass index (BMI), pack-years of smoking, total miRNA counts, and the number of red blood cells (RBCs), white blood cells (WBCs), and platelets; SE indicates standard error.

In Figure 1.2, we show the fold change (95% CI) of four selected evmiRNAs (miR-142-3p, miR-191-5p, miR-199a/b-3p and let-7a-5p), in response to ambient PM\(_{2.5}\) levels at different time windows. These evmiRNAs showed the highest statistical significance in both long-term (6-month and 1-year) PM\(_{2.5}\) time windows, as well as the largest overall fold change. Additionally, we provide the fold change (95% CI) of other evmiRNAs (let-7g-5p, miR-126-3p, miR-15a-5p, miR-223-3p, miR-23a-3p and miR-93-5p) that were significantly associated with both long-term PM\(_{2.5}\) time windows (6-month and 1-year), but were associated with lower overall fold change (Figure S1.2). Finally, we show the longitudinal correlations of all 31 evmiRNAs detected in serum samples from three consecutive visits of 10 participants (n=30) over the sampling period 2000-2008 (Table 1.3). This analysis revealed a weak correlation of the detected evmiRNAs over the period of time that samples were collected.
Figure 1.2: Fold change (95% CI) of selected miRNAs in extracellular vesicles over different time windows of ambient PM$_{2.5}$ levels.

Fold changes (95% CI) for miR-142-3p, miR-191-5p, miR-199a/b-3p, and let-7a-5p in response to ambient PM$_{2.5}$ one-day, one-week, one-month, three-month, six-month, and one-year moving averages before blood sample collection, respectively; all estimates were adjusted for age; body mass index (BMI); number of pack-years of smoking; total miRNA counts, and the number of red blood cells (RBCs), white blood cells (WBCs), and platelets; SD indicates standard deviation.
Table 1.3: Within-individual Spearman’s correlations of miRNAs in serum extracellular vesicles from each visit.

<table>
<thead>
<tr>
<th>miRNA</th>
<th>1st vs. 2nd</th>
<th>2nd vs. 3rd</th>
<th>1st vs. 3rd</th>
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<td>hsa-miR-1246</td>
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<td>0.07</td>
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<td>-0.28</td>
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</table>
miRNA targets, pathway analysis, and biological relevance

To explore the plausible biological function of miRNAs, detected in EVs, that were associated with ambient PM$_{2.5}$ exposures, we included all evmiRNAs (n=16) that reached statistical significance in the adjusted models (Table S1.3) for further in-silico analysis using the IPA software. We found an interaction between seven evmiRNAs (let-7g-5p, miR-126-3p, miR-130a-3p, miR-146a-5p, miR-150-5p, miR-191-5p, and miR-23a-3p) and 16 unique, experimentally validated mRNA targets (CCR3, CD40, COL1A2, CSF1, CXCL12, CXCL8, CXCR4, IL1F10, IL36A, IL36B, IL36G, IL36RN, IL37, IL6, PDGFB, and VCAM1). These interactions were enriched in significant cardiovascular-related pathways such as atherosclerosis, cardiac hypertrophy, and inflammatory responses (Figure 1.3). When we further explored associations between all evmiRNAs and CHD history, we observed an odds ratio (95% CI) of 2.32 (95% CI: 1.33, 4.04; p=0.05) for miR-4454, 2.24 (95% CI: 1.34, 3.75; p=0.07) for miR-720, 0.38 (95% CI: 0.20, 0.74; p=0.04) for miR-130a-3p, and 0.47 (95% CI: 0.27, 0.81; p=0.05) for miR-106b-5p. No significant associations were observed between other evmiRNAs and CHD after adjusting for FDR (Figure S1.3; Table S1.4). Odds ratios for the association between all evmiRNAs and CHD are shown in Figure S1.3 and Table S1.4 (Supplemental Material).
Figure 1.3: Pathway analysis illustrating the link between PM$_{2.5}$-associated miRNAs in extracellular vesicles and biological pathways related to cardiovascular disease.

Selected miRNAs (in red) associated with long-term ambient PM$_{2.5}$ levels and their experimentally validated mRNA targets (in green) as identified by the Ingenuity Pathway Analysis software. The biological pathways involved in cardiovascular disease (CVD) for all miRNA-mRNA interactions are also shown here. Solid lines represent a direct interaction and dotted lines represent an indirect interaction.
DISCUSSION

In this study, we identified an association between 16 evmiRNAs and long-term ambient PM$_{2.5}$ levels. To further investigate their biological function, we conducted *in silico* pathway analysis using the IPA software. We identified seven evmiRNAs (let-7g-5p, miR-126-3p, miR-130a-3p, miR-146a-5p, miR-150-5p, miR-191-5p, and miR-23a-3p) with 16 experimentally validated mRNA targets (CCR3, CD40, COL1A2, CSF1, CXCL12, CXCL8, CXCR4, IL1F10, IL36A, IL36B, IL36G, IL36RN, IL37, IL6, PDGFB, and VCAM1) that are involved in several CVD-related signaling pathways such as atherosclerosis, cardiac hypertrophy, Toll-like receptor, T-Helper cell differentiation, cytokines mediating communication between immune cells, and intercommunication between innate and adaptive immune cells. Exploratory analysis on the association between levels of evmiRNAs and CHD history suggested that overall higher levels of evmiRNAs in the blood circulation were associated with no CHD history. Collectively, these results are consistent with our hypothesis that air pollution is associated with the levels of evmiRNAs circulating in blood, which may participate in biological functions relevant to CVD-related pathways.

Using our newly developed spatio-temporal model to estimate the levels of PM$_{2.5}$, we found significant associations of the levels of certain evmiRNAs with relatively low differences in the concentrations of ambient PM$_{2.5}$. A recent study by Madrigano et al. in the Worcester Heart Attack Study (Worcester, MA), reported a significant 16% increase in the odds of acute myocardial infraction per interquartile range increase (0.59 μg/m$^3$) of PM$_{2.5}$ in a study with a similarly narrow range of 1-year PM$_{2.5}$ exposures (10.44 ± 1.36 μg/m$^3$) (Madrigano et al. 2013). Taken together, these findings provide biological plausibility of the underlying molecular mechanisms in the association of PM$_{2.5}$ exposures and CVD, even at lower concentrations and variation, such as those measured in the Greater Boston area.
The overall increase in evmiRNAs levels associated with PM\textsubscript{2.5} exposures may have originated from either of both of two different biological mechanisms, i.e., an increase in the numbers of EVs released in the blood circulation by blood cells or tissues sensitive to air pollution, and/or an increase in the amount of specific miRNAs loaded in EVs prior to their release in the blood circulation. Bollati et al., showed a similar pattern of an overall increase in the levels of miRNAs detected in EVs after short-term exposure to PM\textsubscript{10} in a group of foundry workers (Bollati et al. 2014). The investigators used an endogenous control to normalize their miRNA expression data which provided additional support to the hypothesis that the overall increase in the detected miRNA levels in response to air pollution might be due to an increase in the selective process of miRNAs loading in EVs, prior to their release in the blood circulation. However, they recognized that the possibility of an increase in the numbers of EVs could have also occurred. Interestingly, recent studies found that elevated numbers of EVs in healthy subjects were linked to increased risk for developing CVD (Chironi et al. 2006; Ueba et al. 2010). Future research is warranted to determine to which extent the overall increase in evmiRNA levels observed in response to PM\textsubscript{2.5} is attributed to increased EV numbers and/or increased EVs miRNA load, prior to their release.

Our findings are consistent with previous \textit{in vitro} and \textit{in vivo} studies showing that miRNA expression in blood cells or other target tissues is sensitive to air pollutants. For instance, in some of our previous work, we demonstrated the differential expression of miR-126-3p, miR-146a-5p, and hsa-let-7g in blood leukocytes in response to short-term PM exposures (Bollati et al. 2010; Fossati et al. 2014; Motta et al. 2013). In addition, a recent \textit{in vivo} study in rats demonstrated a dose-response relationship between exposure to PM and miRNA expression levels in the left ventricle of the heart; among others, miR-19b-3p, miR-146a-5p, miR-150-5p and miR-191-5p were differentially expressed in response to higher doses of PM (Farraj et al. 2011). Furthermore, Fry and colleagues showed that miR-199a/b-3p and miR-223-3p were
upregulated in sputum cells in response to air pollution (Fry et al. 2014). In agreement with these studies, our data show an increase in the levels of miR-126-3p, miR-146a-5p, miR-150-5p, miR-19b-3p, miR-191-5p, miR-199a/b-3p, miR-223-3p, and let-7g-5p in EVs circulating in serum. Further, we herein provide data on several other evmiRNAs sensitive to PM$_{2.5}$ (i.e., miR-142-3p, miR-23a-3p, and miR-93-5p) that have not been reported previously. Additional analysis of the within-individuals long-term correlation of evmiRNAs showed an overall weak correlation. This finding suggests that PM$_{2.5}$-associated evmiRNAs may be more sensitive to between-individual, rather than within-individual, factors and that their change in response to air pollution does not persist in the long-term. For example, Giusti et al. showed that EV release in response to cellular stimulation by fetal bovine serum (FBS) is a time-dependent process in which several EV parameters, including number and composition, are altered (Giusti et al. 2015). Furthermore, Tian et al. revealed that the uptake of EVs by target cells is a very dynamic and fast process (Tian et al. 2013). Thus, we hypothesize that the EVs cargo (i.e., miRNAs) is associated to this dynamic change as well. Collectively, these studies provide evidence in support of our findings for weak long-term correlations between evmiRNAs.

To gain more insights about the mRNA target(s) of each of the PM-associated evmiRNAs and their plausible impact on cellular processes and downstream biological pathways, we utilized the IPA databases. We restricted our analysis to only those miRNA-mRNA interactions that were experimentally validated by either in vitro or in vivo studies. Of the 16 evmiRNAs included in the analysis, seven exhibited biological functional interactions with 16 unique mRNAs enriched in CVD-related pathways such as atherosclerosis, inflammation, and cytokine-mediated pathways. For example, this analysis revealed miRNA-mRNA interactions between miR-146a-5p and critical pro-inflammatory cytokines such as IL-6 and the IL-36 family (IL-36A, IL-36B and IL-36G). miR-146a-5p is an important regulator of IL-6 via the nuclear factor-kappa-B (NF-kB) pathway, and can regulate the expression of NF-kB in macrophages by
targeting the TRAF6 and IRAK1 proteins upstream the NF-kB signaling pathway (Cheng et al. 2013). In turn, IL-6 was found to regulate the expression of the miR-17/92 cluster, including miR-19b-3p, via the activation of STAT3, which can directly target BMPR2, a surface protein receptor that is expressed in endothelial and vascular smooth muscle cells (Brock et al. 2009). The IL-36 family is released by either activated immune cells or epithelial cells and contributes to inflammatory response via the NF-kB pathway (Dietrich et al. 2014), which makes IL-36 targeting a fine-tuner of the NF-kB pathway (Recchiuti et al. 2011). In addition, miR-146a-5p targets TRAF6 and IRAK1, proteins that are part of the CD40 signaling pathway (Donners et al. 2008); CD40, which serves an important role in cellular communication during inflammatory responses, is implicated in atherosclerosis (Schonbeck et al. 2001). IPA also revealed interactions of miR-146a-5p and miR-23a-3p with the chemokines C-X-C motif ligand 8 and 12 (CXCL8 and CXCL12), respectively. Both cytokines have been reported to regulate inflammation (Karin 2010; Maghazachi 2010), and thus their fine-tuning by miRNAs could be critical. Indeed, CXCL8 can be regulated by mir-146a-5p (Yang et al. 2011) and miR-20a-5p (Yu et al. 2010), whereas CXCL12 can be regulated by mir-23a-3p (Lewis et al. 2003). Further, we found an interaction between the endothelial-specific miR-126a-3p and vascular adhesion molecule 1 (VCAM-1), a trans-membrane receptor in endothelial cells. VCAM-1 is expressed by endothelial cells in response to inflammation and plays critical role in recruiting leukocytes at the site of inflammation (Osborn et al. 1989). A study by Harris et al., found that miR-126a-3p can regulate the expression of VCAM-1 in endothelial cells with implications for vascular inflammation and atherosclerosis (Harris et al. 2008). In the same context, we found an interaction between miR-150-5p and the platelet-derived growth factor beta (PDGF-B), a protein that is expressed by smooth muscle endothelial and epithelial cells. PDGF-B plays a central role in cell proliferation and has been implicated in inflammatory responses and atherosclerosis. A study by Shen and colleagues found that miR-150-5p can directly target and regulate the expression of PDGF-B in retina epithelial cells (Shen et al. 2008). Lastly, miR-126a-3p has also
a regulatory role for the expression of CXCL12 via the inhibition of CXCR4 by RGS16, a signaling pathway that is involved in atherosclerosis and inflammation (Wei et al. 2013).

We further explored the association between evmiRNAs and coronary heart disease history in the study participants. We observed some signals of association between certain evmiRNAs and increased (miR-4454 and miR-720) or decreased (miR-106b-5p and miR-130a-3p) odds ratio of CHD history. Overall, PM$_{2.5}$-induced evmiRNAs did not show significant associations with CHD history, except miR-130a-3p; however, we observed that higher levels of PM$_{2.5}$-induced were more likely to be measured in participants with no CHD history. These findings suggest that an adaptive response to long-term PM$_{2.5}$ exposures might be in place. Yamamoto et al., showed that the expression of miR-144-3p in peripheral blood was induced by diesel exposures, and that it was involved in downstream adaptive response pathways to oxidative stress triggered by air pollutants (Yamamoto et al. 2013). In our study, we found that miR-144-3p levels were marginally significantly induced (p=0.06) by long-term PM$_{2.5}$ exposures (Table S1.3) and that higher levels were more likely to be observed in participants with no CHD history [OR: 0.49 (0.24, 0.98); p=0.17] (Figure S1.3; Table S1.4). Our findings are limited by the sample size and use of cross-sectional data, and any inferences on the biological relevance of PM-induced evmiRNAs in the development or progression of CVD-related outcomes must be drawn very carefully. However, we provide data to encourage further research in larger prospective studies to confirm our findings, as well as experimental studies to determine the role of PM$_{2.5}$-induced miRNAs in the development and progression of CVD.

Due to limited sample volume, we were not able to validate the miRNA data with a different platform such as real-time qPCR. However, Knutsen et al. showed an overall high correlation (r=0.703-0.797) when they compared the miRNA fold-change values in NanoString nCounter® and other platforms, including real-time qPCR (Knutsen et al. 2013). For the same reason, we could not quantify the number of EVs, nor we could characterize the different
subpopulations (e.g., based on the tissue of origin) of EVs in the blood of the study participants. It is well documented that the number of circulating EVs in the blood varies between individuals, and that EVs sub-populations can originate from several tissues. In the blood, EVs primarily originate from blood cells (i.e., red blood cells, platelets, and white blood cells), and, to a lesser extent, from endothelial cells, lung epithelial cells, and cardiomyocytes (Yanez-Mo et al. 2015). To address these limitations, we controlled for different cell/tissue sources in the statistical analysis by adjusting for the number of RBCs, WBCs, and platelets. We also controlled for the between-individuals difference in the numbers of EVs by adjusting for the total number of miRNA counts measured in each sample.

We used ultracentrifugation to isolate EVs, which may not prevent contamination from other sources of circulating miRNAs in serum. Arroyo and colleagues showed that miRNAs bound to Argonaute2 (Ago2) protein represent an additional source of circulating miRNAs in blood (Arroyo et al. 2011). Due to their different physical properties, these complexes are not expected to pellet with EVs, but remain in the supernatant fraction after ultracentrifugation. Nonetheless, aggregates of Ago2:miRNA complexes might co-precipitate with EVs in the pellet during isolation. To have some indication on possible contamination in our samples, we compared our miRNA data with the data generated by Arroyo et al. We found that 11 out of the 16 miRNAs that were significantly associated with long-term exposures to PM$_{2.5}$ in our study, were also detected by Arroyo et al. in the EVs (pellet) fractions (let-7a-5p, miR-126-3p, miR-142-3p, miR-146a-5p, miR-150-5p, miR-191-5p, miR-19b-3p, miR-199a/b-3p, miR-23a-3p, miR-223-3p and miR-93-5p). This is particularly encouraging for the validity and efficiency of our method; however, we cannot completely exclude the possibility of minor contamination from co-precipitated Ago2:miRNA complexes. In fact, we identified one miRNA (let-7g-5p) in our data that was found to be exclusively enriched in Ago2 fractions in the Arroyo study. Four miRNAs (miR-1246, miR-130a-3p, miR-15a-5p and miR-505-3p) were not reported in this study due to
technical reasons. A recent study also compared miRNA profiles between cell-free serum, which includes both EVs and miRNA:protein complexes, and EVs isolated from serum by ultracentrifugation; among others, all of the 16 miRNAs that we found to be associated with long-term PM$_{2.5}$ exposures, including miR-1246, miR-15a-5p, miR-130a-3p and miR-505-5p, were detected in EVs (Cheng et al. 2014).

To further explore the origin of the evmiRNAs detected in our study, we searched for tissue-specific miRNA expression studies in the literature. Several miRNAs were found to be highly expressed in cells and tissues that contribute to the total population of EVs in the blood circulation. For example, studies have linked the expression of miR-126-3p to the heart and lung endothelial cells (Wang et al. 2008); miR-142-3p to blood mononuclear cells and the lymphatic system (Landgraf et al. 2007); miR-146a-5p to white blood cells and the respiratory system (Mestdagh et al. 2011); miR-150-5p to the heart, mononuclear blood cells, and the lymphatic system (Landgraf et al. 2007; Mestdagh et al. 2011); miR-199a/b-3p to the heart and blood mononuclear cells (Liang et al. 2007; van Rooij et al. 2006); miR-19b-3p to the heart (Farraj et al. 2011); miR-223-3p to blood cells (Landgraf et al. 2007); and miR-93-5p to heart endothelial cells (Fichtlscherer et al. 2010). Unfortunately, mRNA gene expression data on target tissues (e.g., heart and blood cells) that could provide additional information on the miRNA-mRNA interactions were not obtainable in vivo. To address this challenge, we restricted our in silico pathway analysis to data that were experimentally validated (in vitro or in vivo) and were relevant to the cardiovascular system. These findings support the hypothesis that specific miRNAs detected in EVs might be released in the blood circulation from cells/tissues sensitive to air pollution.

In our study, all participants were non-hispanic white older males with very similar socioeconomic status. We acknowledge that the characteristics of the study participants limit the generalizability of our findings to other populations; however, the homogeneity of the study
participants helped to reduce potential confounding. This study has several other strengths, including the use of hybrid spatio-temporal land-use regression models to estimate the ambient PM$_{2.5}$ levels at the residential address of study participants. Exposure assessment has been a major limitation in many studies; however, our newly developed validated hybrid approach integrates satellite AOD data, land-use variables, weather data, and PM$_{2.5}$ source emissions data from the U.S. EPA to calculate the ambient PM$_{2.5}$ levels. Thus, this method allows us to more accurately and reliably measure PM$_{2.5}$ ambient levels at a 10x10-km resolution. Furthermore, we used a state-of-the-art technology such as the NanoString nCounter® platform to screen for 800 miRNAs. This provides us with the opportunity to screen for a large number of evmiRNAs that have not been investigated in relation to air pollution previously. Lastly, we utilized available data from validated questionnaires and medical records in the NAS including critical covariates considered in the analysis such as age, anthropometric measures, pack-years of smoking, and counts of blood cells.

**CONCLUSIONS**

In summary, we showed that long-term ambient PM$_{2.5}$ levels, is associated with the levels of several evmiRNAs circulating in the blood of older individuals. We found that several of these evmiRNAs are enriched in CVD-related pathways, which may have implications for the association between air pollution and CVD. Further research is warranted to replicate our findings in larger and more diverse populations, as well as determine the role of air pollution-induced evmiRNAs in human health.
ABBREVIATIONS

AOD: Aerosol Optical Density
BH: Benjamini-Hochberg
BMI: Body Mass Index
CHD: Coronary Heart Disease
CI: Confidence Interval
CRP: C-reactive Protein
CVD: Cardiovascular Disease
CXCL: C-X-C Motif Ligand
EDTA: Ethylenediaminetetraacetic Acid
EVs: Extracellular Vesicles
FDR: False Discovery Rate
IL: Interleukin
IPA: Ingenuity Pathway Analysis
NAS: Normative Aging Study
NF-kB: Nuclear Factor-kappa-B
PBL: Planetary Boundary Layer
PDGF-B: Platelet-Derived Growth Factor Beta
PM: Particulate Matter
RBCs: Red Blood Cells
RISC: RNA-Induced Silencing Complex
SD: Standard Deviation
US EPA: United States Environmental Protection Agency
VCAM-1: Vascular Adhesion Molecule 1
WBCs: White Blood Cells
CONFLICT OF INTEREST

The authors declare that they have no competing interests.

AUTHORS’ CONTRIBUTIONS

RR and AAB developed the study concept. RR designed and performed the experiments, analyzed the data, and wrote the manuscript. PV contributed participants’ material. JS developed estimates of ambient particulate air pollution levels. BC, QL, JS, and PV provided critical revisions. AAB conceived the study, oversaw research, helped to write the manuscript, and provided intellectual input throughout the study. All authors have read and approved the final manuscript.

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[https://www.epa.gov/air-emissions-inventories](https://www.epa.gov/air-emissions-inventories).


Table S1.1: Characteristics of study participants at first examination (n=22).

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<th>Characteristic</th>
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<tr>
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<tr>
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</tr>
<tr>
<td>Current</td>
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</tr>
<tr>
<td>Former†</td>
<td>16 (62.8)</td>
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<tr>
<td>Pack-years of smoking</td>
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<td>Race; n(%)</td>
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<tr>
<td>Non-hispanic white</td>
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</tr>
<tr>
<td>Coronary heart disease; n(%)</td>
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</tr>
<tr>
<td>Platelets (thousands/mm³)</td>
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</tr>
<tr>
<td>Red blood cells (thousands/mm³)</td>
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</tr>
<tr>
<td>White blood cells (thousands/mm³)</td>
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</tr>
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*Mean ± SD, unless otherwise specified.
†Former smokers quit smoking at least 12 years prior to enrollment.

Table S1.2 (available online): Univariate association of ambient PM$_{2.5}$ levels and all measured miRNAs (n=798) in extracellular vesicles (EVs).
**Table S1.3**: Associations between ambient PM$_{2.5}$ moving average time windows and levels of miRNAs in extracellular vesicles.

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| miR-720      | -0.33         | 0.25| 0.44                 |
| miR-4454     | -0.29         | 0.22| 0.44                 |
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<tr>
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<td>miR-25-3p</td>
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<tr>
<td>miR-720</td>
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<tr>
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<tr>
<td>miR-320e</td>
<td>0.004</td>
<td>1.00</td>
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</table>

1 Regression coefficient estimating the effect on ex-miRNA levels (log$_2$) for every SD increase in PM$_{2.5}$ levels; models adjusted for age, BMI, pack-years of smoking, RBC, WBC and PLT.
Table S1.4: Odds ratios (95% CI) of the association between miRNAs in extracellular vesicles and coronary heart disease history.

<table>
<thead>
<tr>
<th>miRNA</th>
<th>OR†</th>
<th>95% CI</th>
<th>FDR adjusted P value</th>
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<tr>
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<td>0.38</td>
<td>(0.20, 0.74)</td>
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<td>miR-4454</td>
<td>2.32</td>
<td>(1.33, 4.04)</td>
<td>0.05</td>
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<td>miR-106b-5p</td>
<td>0.47</td>
<td>(0.27, 0.81)</td>
<td>0.05</td>
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<tr>
<td>miR-720</td>
<td>2.24</td>
<td>(1.34, 3.75)</td>
<td>0.07</td>
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<td>miR-23a-3p</td>
<td>0.61</td>
<td>(0.39, 0.94)</td>
<td>0.16</td>
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<tr>
<td>miR-30d-5p</td>
<td>0.57</td>
<td>(0.34, 0.95)</td>
<td>0.16</td>
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<td>miR-142-3p</td>
<td>0.56</td>
<td>(0.33, 0.97)</td>
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<tr>
<td>miR-144-3p</td>
<td>0.49</td>
<td>(0.24, 0.98)</td>
<td>0.17</td>
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<td>0.49</td>
<td>(0.22, 1.07)</td>
<td>0.25</td>
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<td>miR-223-3p</td>
<td>0.68</td>
<td>(0.40, 1.14)</td>
<td>0.36</td>
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<tr>
<td>miR-15a-5p</td>
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<td>(0.40, 1.19)</td>
<td>0.37</td>
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<td>miR-146a-5p</td>
<td>0.84</td>
<td>(0.65, 1.08)</td>
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<td>0.38</td>
</tr>
<tr>
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<td>(0.32, 1.15)</td>
<td>0.39</td>
</tr>
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<td>miR-320e</td>
<td>0.60</td>
<td>(0.29, 1.24)</td>
<td>0.40</td>
</tr>
<tr>
<td>miR-16-5p</td>
<td>0.73</td>
<td>(0.44, 1.20)</td>
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<tr>
<td>miR-126-3p</td>
<td>0.78</td>
<td>(0.51, 1.17)</td>
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<td>0.86</td>
<td>(0.65, 1.13)</td>
<td>0.47</td>
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<td>miR-1246</td>
<td>1.09</td>
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<td>miR-191-5p</td>
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<tr>
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<td>(0.64, 1.27)</td>
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<td>(0.63, 1.32)</td>
<td>0.84</td>
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<td>0.96</td>
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<td>miR-342-3p</td>
<td>1.04</td>
<td>(0.65, 1.66)</td>
<td>0.97</td>
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<tr>
<td>miR-181a-5p</td>
<td>1.02</td>
<td>(0.79, 1.32)</td>
<td>0.98</td>
</tr>
<tr>
<td>miR-451a</td>
<td>1.10</td>
<td>(0.47, 2.57)</td>
<td>0.99</td>
</tr>
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<td>miR-185-5p</td>
<td>1.00</td>
<td>(0.27, 3.68)</td>
<td>1.00</td>
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</table>

†All models were adjusted for age, body mass index (BMI), pack-years of smoking, total miRNA counts, and the number of red blood cells (RBCs), white blood cells (WBCs), and platelets.
**Figure S1.1. Morphological characterization of serum extracellular vesicles (EVs).**

Preparations of EVs were imaged by transmission electron microscopy (TEM). (a) non-labeled EVs, (b) EVs labeled with gold-conjugated anti-CD63 antibody, (c) EVs labeled with gold-conjugated anti-CD81 antibody. Images were taken by a JEOL 1200EX microscope coupled with an AMT 2k CCD camera, at the Harvard Medical School Electron Microscopy Core.
Figure S1.2. Fold change (95% CI) of selected miRNAs in extracellular vesicles over different time windows of ambient PM$_{2.5}$ levels.

Fold changes (95% CI) for let-7g-5p, miR-126-3p, miR-15a-5p, miR-223-3p, miR-23a-3p and miR-93-5p in response to ambient PM$_{2.5}$ one-day, one-week, one-month, three-month, six-month, and one-year moving averages before blood sample collection, respectively; all estimates were adjusted for age; body mass index (BMI); number of pack-years of smoking; total miRNA counts, and the number of red blood cells (RBCs), white blood cells (WBCs), and platelets; SD indicates standard deviation.
Figure S1.3. Forest plot showing odds ratios (95% CI) of the association between miRNAs in extracellular vesicles and coronary heart disease history.

All estimates were adjusted for age; body mass index (BMI); number of pack-years of smoking; total miRNA counts, and the number of red blood cells (RBCs), white blood cells (WBCs), and platelets.
CHAPTER 2

Prenatal Lead Exposure and Birthweight Association: Are Smaller Infants More Susceptible?

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ABSTRACT

Background

As population lead levels decrease, the toxic effects of lead may be distributed to more sensitive populations, such as infants with poor growth.

Methods

We examined the association of second trimester maternal blood lead levels (BLL) with birthweight-for-gestational age (BWGA) z-score in a Mexican cohort of 946 mother-child pairs. We determined the association between maternal BLL and BWGA z-score by using both linear and quantile regression models. We further estimated the odds ratio for small-for-gestational age (SGA) infants between quartiles of maternal BLL using logistic regression models. We selected maternal age, body mass index (BMI), socioeconomic status (SES), parity, household smoking exposure, hemoglobin levels and infant sex a priori as potential confounders.

Results

The mean ± SD second trimester maternal BLL was 3.7 ± 2.7 μg/dL. While linear regression showed a negative association between maternal BLL and BWGA z-score (-0.06 z-score units per log₂ BLL increase; 95% CI: -0.13, 0.003; p=0.06), quantile regression revealed a larger and significant magnitude of the association (range: -0.08, -0.13 z-score units per log₂ BLL increase); all p-values <0.05 in the lower BWGA z-score percentiles (<30th percentile), including SGA infants (<10th percentile). Mothers in the highest BLL quartile had an odds ratio of 1.62 (95% CI: 0.99-2.65) for having a SGA infant as compared to the lowest BLL quartile.
Conclusions

Quantile regression revealed a negative association between prenatal exposure to lead and birthweight and suggested that smaller infants represent a susceptible subpopulation. This effect would have been missed by traditional linear regression.

**Key words:** Lead; Prenatal Exposure; Birthweight; Small-For-Gestational Age; Pregnancy; Quantile Regression;
BACKGROUND

Poor fetal growth precedes ~60% of neonatal deaths (Black et al. 2008) and leads to adverse fetal growth outcomes such as low birthweight and small-for-gestational age (SGA) (Victora et al. 2008). Low birthweight (<2,500 grams) and SGA (<10th percentile of the birthweight-for-gestational age distribution) infants, term and preterm, have an increased risk of chronic developmental and cardiometabolic disorders later in life (Lawn et al. 2014), and impose a substantial socioeconomic burden worldwide (Bhutta et al. 2014). Globally, the prevalence of low birthweight is estimated to be 20 million infants and of SGA about 32 million infants (of whom ~10 million are term low birthweight), with particularly high prevalence in low and middle income countries (Lee et al. 2013). Numerous preventable risk factors have been linked to poor fetal growth, including prenatal exposure to environmental lead (Jelliffe-Pawlowski et al. 2006).

Lead is a toxic heavy metal that is widespread in the environment. Exposure to lead in developed countries has dropped dramatically in the last 30 years; however, toxic effects are still reported even in populations with blood lead levels once believed to be safe (i.e. <5 µg/dL). During pregnancy, maternal lead can easily cross the placenta and enter the fetal blood circulation (Lin et al. 1998). Due to similar physical and chemical properties, lead competes with calcium for deposition into the bone, which might lead to impaired fetal growth (Potula et al. 2005). Lead also binds to sulfhydryl groups and inhibits multiple enzymes such as those involved in heme synthesis, which is critical to P450 enzymes involved in cellular respiration, metabolism as well as hemoglobin synthesis (Flora et al. 2012). Several epidemiological studies have shown an inconsistent association of prenatal lead exposure and fetal growth (Nishioka et al. 2014; Zhang et al. 2015; Jelliffe-Pawlowski et al. 2006; Zhu et al. 2010; Cantonwine et al. 2010; Gonzalez-Cossio et al. 1997; Burris et al. 2011; Wigle et al. 2007). These studies used traditional regression methods, also known as ordinary least squares (OLS) regression, to estimate the conditional mean response of the association between exposure to lead and fetal
growth. However, OLS methods are limited in capturing differences in the magnitude and direction of the association between a predictor (e.g., prenatal exposure to lead) and the outcome (e.g., birthweight) across the entire distribution of the outcome (Koenker 2005).

The value of quantile regression, which allows for effects of the exposure (i.e., lead) to vary across the distribution of the outcome (i.e., birthweight) has been demonstrated previously in lead poisoning with regards to school performance. Miranda et al., used quantile regression to show that childhood lead exposure is predicted poorer on standardized state test scores in school age children with more pronounced effects in the low end of the test score distribution (Burgette et al. 2011). We build upon this research by testing whether lead exposure in pregnancy predicts smaller birthweight more prominently at the lower range of the birthweight-for-gestational age (BWGA) distribution. In other words, are children with poor fetal growth more susceptible to lead toxicity than children with normal growth patterns? Fetal growth is a complex and dynamic process, with infants at the tails of the outcome (e.g., birthweight) distribution suffering a disproportionate burden of perinatal morbidities. We hypothesized that OLS regression analysis may imprecisely estimate associations that occur in the tails of the birthweight distribution, and that these associations could be revealed by using quantile regression models.

Lead exposure is still a major public health problem in Mexico (Caravanos et al. 2014). The prevalence of low birthweight and small-for-gestational age (SGA), which are measures of poor fetal growth, is relatively high (~10%) in the Mexican population (Lee et al. 2013). Therefore, we used data from the follow up of the Programming Research in Obesity, GRowth Environment and Social Stress (PROGRESS) prospective cohort study to measure second trimester blood lead levels in 946 pregnant women in Mexico City, and determine the association between prenatal lead exposure and fetal growth as measured by BWGA z-score and risk of SGA.
METHODS

Study population

Study participants were enrolled as part of the PROGRESS birth cohort project, in Mexico City, Mexico, between 2007 and 2011. Details of the cohort’s profile and enrollment are described elsewhere (Braun et al. 2014; Burris et al. 2013). In brief, pregnant women who attended the Mexican Social Security Institute (Instituto Mexicano del Seguro Social) clinics for their prenatal care were enrolled. Eligibility criteria for participation in the study were gestational age <20 weeks, maternal age of ≥18 years, planned to live in Mexico City for the next three years, and had access to a telephone. Exclusion criteria were chronic medical conditions such as heart or kidney disease; use of steroids or anti-epilepsy drugs; daily consumption of alcoholic beverages; and drug addiction. We screened 3,898 women who presented to the IMSS clinic during this time and enrolled 1054 who met the eligibility criteria and provided written informed consent to participate. Of these, 946 gave birth to a live infant and remained in the study. This represents our base population. Two participants had missing blood lead measurements and so they were excluded resulting to the number of 944 participants included in the statistical analysis. A flow diagram of the study is shown in Figure S2.1. The study was approved by the Institutional Review Boards of Brigham and Women’s Hospital (#14265-101; #14706-101; #2006-P-001416; #2006-P-001792) and the National Institute of Public Health in Mexico (#560) according to the declaration of Helsinki.

Data collection

Baseline information on demographics, anthropometric characteristics and health status was collected from all participants at first visit (12-24 weeks of gestation), and at every subsequent visit until delivery. Due to the lack of ultrasound measurements, gestational age was calculated
using the date of last menstrual period (LMP) and by an additional standardized physical
examination assessment at birth (Capurro et al. 1978). In cases where the estimated gestational
age differed by more than three weeks between the two methods (~4% of the study
participants), the physical examination assessment data were used. Second trimester (12-24
weeks of gestation) maternal blood lead levels were used, which was the earliest time point lead
was measured. Infants’ birthweight was adjusted for gestational age (BWGA) at delivery, and
percentiles and z-scores were calculated according to the international infant growth charts
developed by Fenton et al. (Fenton et al. 2013). We defined infants with a BWGA z-score <10th
percentile as small-for-gestational age. Socioeconomic status (SES) was calculated based on a
hierarchical model including the following 13 variables: (1) education of the head of household,
(2) number of rooms, (3) number of bathrooms with showers, (4) type of floor, (5) number of
light bulbs and ownership of (6) car, (7) hot water heater, (8) automatic washing machine, (9)
videocassette recorder, (10) toaster, (11) vacuum cleaner, (12) microwave oven, and (13)
personal computer (Carrasco 2002). Other lifestyle information such as alcohol consumption
and household smoking exposure were also attained from the validated in-person questionnaire
at first visit.

**Measurement of blood lead levels**

Blood samples were collected in trace metal-free tubes at second trimester (12-24 weeks of
gestation) and stored at -20°C until further analysis. Upon thaw on ice, blood samples (1mL)
were weighed, digested in 2 mL of ultra-pure concentrated HNO₃ acid (1mL) for 48 hours and
then diluted to 10 mL with deionized water after the addition 0.5 ml of 30% hydrogen peroxide.
Sample handling was performed in ISO Class 5 laminar flow clean hood in an ISO Class
6 clean room. Digested samples were analyzed using external calibration with seven calibration
points using an Agilent 8800 ICP Triple Quad (ICP-QQQ) instrument (Agilent technologies, Inc.,
Delaware, USA) in MS/MS mode with Lutetium as the internal standard. Quality control (QC) and quality assurance (QA) procedures included analyses of calibration verification standards and continuous calibration verification standards (CCVS) [1 ng/mL and 5 ng/mL standards, National Institute of Standard and Technology Standard Reference Material (NIST SRM) 1640a (trace elements in natural water, Gaithersburg, MD)], procedural blanks, duplicates, spiked samples, NIST SRM 955c (Toxic metals in Caprine blood), Seronorm, Trace Elements Whole Blood L-3 (SERO, Billingstad, Norway), and in house pooled blood sample (IHB) to monitor the accuracy, recovery rates and reproducibility of the procedure for each analytic batch. CCVS and IHB were run after analysis of every 10 samples. All laboratory recovery rates for QC standards and spiked samples with this method were 85 to 115% and precision (given as % relative standard deviation) is <10% for samples with concentrations > limit of quantitation. The limit of detection for lead by this procedure was 0.02 ng/mL and limit of quantitation was 0.07 ng/mL.

**Statistical analysis**

We quantified descriptive characteristics of the study participants at second trimester using mean ± standard deviation (SD). To improve normality in our data, we log-transformed (log₂) the maternal BLL. We used linear regression models to estimate the association between maternal BLL (continuous) and BWGA z-score (continuous). To adjust for the association between maternal BLL and BWGA z-score, we selected *a priori* the following covariates: age (continuous), body mass index (BMI) (continuous), SES (low, medium, and high), hemoglobin levels (continuous), and infant sex. Other covariates such as parity, alcohol consumption, and household smoking exposure were also considered in the analysis; however, they were not included in the final models as they did not confound the association between maternal BLL and BWGA z-score (assessed by a change of >10% of the $\beta$ coefficient estimate), and did not improve the fit of the model (assessed by the model’s $R^2$ value); therefore, they were dropped.
from the final models. To further investigate the association between maternal BLL and BWGA z-score, we used quantile regression models adjusting for the same covariates as in the linear regression models. We used the \( \beta \) coefficient estimates from both linear and quantile regression models to calculate the study population’s distributional shift in birthweight-for-gestational age associated with a \( \log_2 \) increase in maternal BLL. Using the same estimates, we calculated the predicted birthweight-for-gestational age distributions under five maternal BLL exposure (\( \log_2 \)) increments. Last, we used logistic regression models to determine the association [odds ratio (OR) and 95% confidence intervals (CIs)] between maternal BLL and small-for-gestational age, and we tested for trend using the median value of the maternal BLL quartiles. All statistical analyses were performed using SAS version 9.4 (SAS Institute Inc., Cary, NC).

RESULTS

The demographics of the study participants are shown in Table 2.1. The mean ± SD second trimester maternal BLL was 3.7 ± 2.7 \( \mu \)g/dL (median 2.8, IQR: 1.9-4.5, range: 0.5-22.9 \( \mu \)g/dL), with 21% of the participants with BLL above the reference level (>5 \( \mu \)g/dL) for pregnant women recommended by the U.S. Centers for Disease Control and Prevention (CDC) 2010). When we \( \log_2 \)-transformed the maternal BLL data, the range spanned through five \( \log_2 \) unit increments. Women with higher BLL were more likely of lower socioeconomic status, older in age, shorter in height, and with elevated levels of hemoglobin (all \( p \)-values <0.05). The mean ± SD infant BWGA z-score was -0.5 ± 0.9, and 165 infants (17.5%) were small-for-gestational age.
### Table 2.1: Characteristics of study participants at second trimester visit (12-24 weeks).

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>N=944 Mean ± SD</th>
<th>Q1 1.93-2.79</th>
<th>Q2 2.80-4.53</th>
<th>Q3 &gt;4.53</th>
<th>p-value&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal Age (years)</td>
<td>27.2 ± 5.5</td>
<td>26.7 ± 5.6</td>
<td>27.0 ± 5.4</td>
<td>27.5 ± 5.7</td>
<td>0.008</td>
</tr>
<tr>
<td>Maternal Height (cm)</td>
<td>155.1 ± 5.5</td>
<td>154.9 ± 5.9</td>
<td>155.5 ± 5.6</td>
<td>154.7 ± 5.4</td>
<td>0.003</td>
</tr>
<tr>
<td>Maternal Body weight (kg)</td>
<td>64.7 ± 4.1</td>
<td>64.6 ± 4.2</td>
<td>65.2 ± 4.1</td>
<td>64.2 ± 4.1</td>
<td>0.29</td>
</tr>
<tr>
<td>Maternal Body mass index (BMI) (kg/m&lt;sup&gt;2&lt;/sup&gt;)</td>
<td>26.9 ± 4.2</td>
<td>26.8 ± 4.3</td>
<td>26.9 ± 4.1</td>
<td>26.6 ± 4.1</td>
<td>0.84</td>
</tr>
<tr>
<td>Maternal Hemoglobin levels (g/dL)</td>
<td>12.9 ± 0.9</td>
<td>12.8 ± 0.8</td>
<td>12.9 ± 0.8</td>
<td>13.0 ± 0.9</td>
<td>0.006</td>
</tr>
<tr>
<td>Maternal Blood lead levels (μg/dL)</td>
<td>3.7 ± 2.7</td>
<td>1.4 ± 0.3</td>
<td>2.4 ± 0.2</td>
<td>3.6 ± 0.5</td>
<td>0.08</td>
</tr>
<tr>
<td>Maternal Multiparous; n (%)</td>
<td>591 (62.6)</td>
<td>141 (14.9)</td>
<td>151 (16.0)</td>
<td>149 (15.8)</td>
<td>0.68</td>
</tr>
<tr>
<td>Maternal Alcohol consumption; n (%)</td>
<td>78 (8.3)</td>
<td>18 (7.6)</td>
<td>22 (9.3)</td>
<td>17 (7.2)</td>
<td>0.38</td>
</tr>
<tr>
<td>Maternal Socioeconomic status (SES); n (%)</td>
<td>484 (51.3)</td>
<td>108 (11.5)</td>
<td>121 (12.8)</td>
<td>120 (12.7)</td>
<td>0.009</td>
</tr>
<tr>
<td>Maternal Low</td>
<td>354 (37.5)</td>
<td>96 (10.2)</td>
<td>86 (9.1)</td>
<td>92 (9.7)</td>
<td>0.84</td>
</tr>
<tr>
<td>Maternal Medium</td>
<td>106 (11.2)</td>
<td>32 (3.4)</td>
<td>29 (3.1)</td>
<td>24 (2.5)</td>
<td>0.22</td>
</tr>
<tr>
<td>Maternal High</td>
<td>295 (31.3)</td>
<td>64 (27.5)</td>
<td>80 (34.0)</td>
<td>81 (34.6)</td>
<td>0.79</td>
</tr>
<tr>
<td>Infant Household smoking exposure&lt;sup&gt;d&lt;/sup&gt;; n (%)</td>
<td>295 (31.3)</td>
<td>64 (27.5)</td>
<td>80 (34.0)</td>
<td>81 (34.6)</td>
<td>0.79</td>
</tr>
<tr>
<td>Infant Birthweight (g)</td>
<td>3043 ± 482</td>
<td>3032 ± 497</td>
<td>3063 ± 439</td>
<td>3024 ± 510</td>
<td>0.008</td>
</tr>
<tr>
<td>Infant Gestational age (weeks)</td>
<td>38.3 ± 1.9</td>
<td>38.1 ± 1.9</td>
<td>38.4 ± 1.8</td>
<td>38.2 ± 2.1</td>
<td>0.84</td>
</tr>
<tr>
<td>Infant Birthweight-for-gestational age&lt;sup&gt;b&lt;/sup&gt; (z-score)</td>
<td>-0.5 ± 0.9</td>
<td>-0.4 ± 0.8</td>
<td>-0.5 ± 0.9</td>
<td>-0.5 ± 0.9</td>
<td>0.79</td>
</tr>
<tr>
<td>Infant Small-for-gestational age (SGA)</td>
<td>165 (17.5)</td>
<td>33 (14.0)</td>
<td>41 (17.4)</td>
<td>43 (18.2)</td>
<td>0.23</td>
</tr>
<tr>
<td>Infant Infant sex; n (%)</td>
<td>498 (52.7)</td>
<td>119 (52.7)</td>
<td>127 (53.8)</td>
<td>124 (52.5)</td>
<td>0.79</td>
</tr>
</tbody>
</table>

<sup>a</sup>All values are shown as mean ± SD, unless otherwise noted.

<sup>b</sup>Birthweight-for-gestational age z-score is adjusted for gestational age and calculated based on the Fenton growth charts.

<sup>c</sup>p-value corresponds to the univariate association between birthweight-for-gestational age z-score and variables considered in the analysis.

<sup>d</sup>Data are missing for seven participants.
In the multivariable linear regression analysis (model 1) adjusted for maternal age, BMI, SES, and infant sex, we found a significant association between second trimester blood lead and BWGA z-score (-0.07, 95% CI: -0.13, -0.003; p=0.04) (Table 2.2). When we further adjusted for hemoglobin (model 2), the association between second trimester blood lead and BWGA z-score was -0.06 (95% CI: -0.13, 0.003; p=0.06). Further analysis using quantile regression adjusted for maternal age, BMI, SES, infant sex, and hemoglobin, confirmed the negative association between maternal BLL and BWGA z-score, and revealed that the association was stronger at the lowest (<30th) z-score percentiles (Table 2.3).

Table 2.2: Association between maternal blood lead levels (per log\textsubscript{2} increment) and birthweight-for-gestational age (z-score) from linear regression.

<table>
<thead>
<tr>
<th>Model</th>
<th>Difference in z-score</th>
<th>SE</th>
<th>95% CI</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unadjusted</td>
<td>-0.06</td>
<td>0.03</td>
<td>(-0.12, 0.007)</td>
<td>0.08</td>
</tr>
<tr>
<td>Model 1\textsuperscript{a}</td>
<td>-0.07</td>
<td>0.03</td>
<td>(-0.13, -0.003)</td>
<td>0.04</td>
</tr>
<tr>
<td>Model 2\textsuperscript{b}</td>
<td>-0.06</td>
<td>0.03</td>
<td>(-0.13, 0.003)</td>
<td>0.06</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Model 1: adjusted for maternal age, body mass index (BMI), socioeconomic status (SES) and infant sex.

\textsuperscript{b}Model 2: Model 1 with additional adjustment for maternal hemoglobin levels.
Table 2.3: Quantile regression analysis of the association of maternal blood lead levels (per $\log_2$ increment) and birthweight-for-gestational age (z-score).

<table>
<thead>
<tr>
<th>Quantile level</th>
<th>Fenton percentile</th>
<th>Difference in z-score$^a$</th>
<th>95% CI</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.05</td>
<td>0.03</td>
<td>-0.08</td>
<td>(-0.19, 0.03)</td>
<td>0.17</td>
</tr>
<tr>
<td>0.10</td>
<td>0.05</td>
<td>-0.13</td>
<td>(-0.25, -0.004)</td>
<td>0.04</td>
</tr>
<tr>
<td>0.15</td>
<td>0.08</td>
<td>-0.11</td>
<td>(-0.22, -0.002)</td>
<td>0.05</td>
</tr>
<tr>
<td>0.20</td>
<td>0.12</td>
<td>-0.12</td>
<td>(-0.20, -0.03)</td>
<td>0.007</td>
</tr>
<tr>
<td>0.25</td>
<td>0.15</td>
<td>-0.10</td>
<td>(-0.19, -0.02)</td>
<td>0.01</td>
</tr>
<tr>
<td>0.30</td>
<td>0.19</td>
<td>-0.11</td>
<td>(-0.18, -0.04)</td>
<td>0.003</td>
</tr>
<tr>
<td>0.35</td>
<td>0.23</td>
<td>-0.04</td>
<td>(-0.12, 0.04)</td>
<td>0.32</td>
</tr>
<tr>
<td>0.40</td>
<td>0.27</td>
<td>-0.06</td>
<td>(-0.14, 0.03)</td>
<td>0.19</td>
</tr>
<tr>
<td>0.45</td>
<td>0.30</td>
<td>-0.05</td>
<td>(-0.13, 0.04)</td>
<td>0.26</td>
</tr>
<tr>
<td>0.50</td>
<td>0.35</td>
<td>-0.07</td>
<td>(-0.16, 0.01)</td>
<td>0.10</td>
</tr>
<tr>
<td>0.55</td>
<td>0.40</td>
<td>-0.07</td>
<td>(-0.16, 0.01)</td>
<td>0.10</td>
</tr>
<tr>
<td>0.60</td>
<td>0.45</td>
<td>-0.07</td>
<td>(-0.15, 0.01)</td>
<td>0.08</td>
</tr>
<tr>
<td>0.65</td>
<td>0.50</td>
<td>-0.04</td>
<td>(-0.12, 0.04)</td>
<td>0.32</td>
</tr>
<tr>
<td>0.70</td>
<td>0.56</td>
<td>-0.04</td>
<td>(-0.12, 0.03)</td>
<td>0.27</td>
</tr>
<tr>
<td>0.75</td>
<td>0.61</td>
<td>-0.01</td>
<td>(-0.08, 0.06)</td>
<td>0.80</td>
</tr>
<tr>
<td>0.80</td>
<td>0.68</td>
<td>-0.02</td>
<td>(-0.1, 0.06)</td>
<td>0.64</td>
</tr>
<tr>
<td>0.85</td>
<td>0.74</td>
<td>-0.06</td>
<td>(-0.16, 0.04)</td>
<td>0.23</td>
</tr>
<tr>
<td>0.90</td>
<td>0.85</td>
<td>-0.07</td>
<td>(-0.16, 0.02)</td>
<td>0.15</td>
</tr>
<tr>
<td>0.95</td>
<td>1.00</td>
<td>-0.02</td>
<td>(-0.13, 0.09)</td>
<td>0.77</td>
</tr>
</tbody>
</table>

$^a$Adjusted for age, body mass index (BMI), socioeconomic status (SES), infant sex and maternal hemoglobin levels.
The effect estimates from quantile regression showed different magnitudes of the association between second trimester maternal BLL and BWGA z-scores across the distribution. We found a substantially larger left shift and change of the shape of the predicted BWGA distribution per log₂ increase in maternal BLL in the lower tail of the distribution (Figure 2.1). For example, while in linear regression analysis a log₂ increase in maternal BLL was associated with a mean [95% confidence interval (CI)] z-score difference of -0.06 (95% CI: -0.13, 0.003; Table 2.2), in quantile regression the estimates were -0.12 (95% CI: -0.20, -0.03; Table 2.3) z-score difference in the 20th percentile and -0.02 (95% CI: -0.1, 0.06; Table 2.3) z-score difference in the 80th percentile of the BWGA distribution.

In the multivariable logistic regression analysis adjusted for maternal age, BMI, SES, and infant sex (model 1), the odds ratio (OR) (95% CI) of SGA for the higher lead exposure quartiles was 1.33 (0.81-2.20) for Q2, 1.40 (0.85-2.31) for Q3, and 1.68 (1.03-2.75) for Q4, as compared to lowest quartile (Q1) of the maternal BLL (Table 2.4). When we further adjusted for hemoglobin (model 2) the OR (95% CI) of SGA for the higher lead exposure quartiles was 1.30 (0.79-2.15) for Q2, 1.37 (0.83-2.25) for Q3, and 1.62 (0.99-2.65) for Q4, as compared to lowest quartile (Q1) of the maternal BLL. Last, we found a marginally significant linear dose-response relationship between prenatal lead exposure at second trimester and increased odds for having a SGA infant (p for trend <0.06).
Figure 2.1: Empirical distribution of birthweight-for-gestational age and associated predicted distributions assuming a log$_2$ increment in maternal blood lead levels.

This figure indicates the empirical (solid line) and predicted (dashed and dotted lines) distributions from the association of prenatal exposure to lead and BWGA z-score as calculated by both linear (top) and quantile (bottom) regression models assuming a range of five increments (log$_2$) in maternal blood lead levels. Quantile regression estimates revealed that prenatal exposure to lead is associated with a heterogeneous shift to left of the birthweight-for-gestational age (BWGA) z-score distribution, increasing in particular the probabilities of lower BWGA z-scores among infants prenatally exposed to higher levels of lead.
Table 2.4: Odds ratios (95 % confidence interval) of small-for-gestational age infants across different quantiles of maternal blood lead levels.

<table>
<thead>
<tr>
<th>Maternal blood lead levels (μg/dL)</th>
<th>Cases/total</th>
<th>Q1 (&lt;1.93)</th>
<th>Q2 (1.93-2.79)</th>
<th>Q3 (2.80-4.53)</th>
<th>Q4 (&gt;4.53)</th>
<th>P for trend</th>
</tr>
</thead>
<tbody>
<tr>
<td>Q1 (&lt;1.93)</td>
<td>33/229</td>
<td>1.28 (0.78-2.10)</td>
<td>1.37 (0.84-2.24)</td>
<td>1.58 (0.97-2.56)</td>
<td>0.07</td>
<td></td>
</tr>
<tr>
<td>Q2 (1.93-2.79)</td>
<td>41/231</td>
<td>1.33 (0.81-2.20)</td>
<td>1.40 (0.85-2.31)</td>
<td>1.68 (1.03-2.75)</td>
<td>0.04</td>
<td></td>
</tr>
<tr>
<td>Q3 (2.80-4.53)</td>
<td>43/229</td>
<td>1.30 (0.79-2.15)</td>
<td>1.37 (0.83-2.25)</td>
<td>1.62 (0.99-2.65)</td>
<td>0.06</td>
<td></td>
</tr>
<tr>
<td>Q4 (&gt;4.53)</td>
<td>48/228</td>
<td>1.38 (0.81-2.31)</td>
<td>1.40 (0.85-2.31)</td>
<td>1.68 (1.03-2.75)</td>
<td>0.04</td>
<td></td>
</tr>
</tbody>
</table>

Model 1: adjusted for maternal age, body mass index (BMI), socioeconomic status (SES) and infant sex.

Model 2: Model 1 with additional adjustment for maternal hemoglobin levels.

DISCUSSION

We found a negative association of prenatal exposure to lead at the second trimester with birthweight-for-gestational age. Quantile regression revealed different magnitudes of the association across the birthweight-for-gestational age distribution, suggesting that susceptibility to lead varied based on differences in fetal growth. In particular, the association was larger for the lowest percentiles of the birthweight-for-gestational age distribution indicating that small-for-gestational age newborns may be more sensitive to lead exposure. Similarly, consistent with our findings from linear and quantile regression, we found that prenatal exposure to elevated maternal blood lead levels was associated with higher odds of small-for-gestational-age birth. Trend test suggests a linear dose-response relationship with no safe threshold for lead exposure.

These findings are consistent with studies in other populations that examined the association between prenatal lead exposure and birthweight. Using linear regression estimates and the international infant growth charts by Fenton et al., we estimated a decrease in birthweight for a 39-week male infant equal to 25 g per log₂, or 10 g per 1μg/dL, increment in
maternal BLL. Recently, Taylor et al. showed a comparable decrease in birthweight (13 g per 1 μg/dL increase in maternal BLL) of infants in a UK cohort of 4,285 infants whose mothers had second trimester maternal BLL of 3.67 ± 1.47 μg/dL (mean ± SD) (Taylor et al. 2015). In addition, Zhu et al., reported a decrease in birthweight of 4-27 g per 1 μg/dL in a cohort of 43,288 mother-infant pairs in New York, USA, with second trimester maternal BLL <10 μg/dL (Zhu et al. 2010).

Similar to our results from linear regression analysis, quantile regression revealed a negative association between prenatal exposure to lead and birthweight-for-gestational age. Quantile regression analysis showed stronger associations at the lower tail of the BWGA distribution, whereas in higher percentiles the magnitude and statistical significance were considerably attenuated. According to our estimates from linear regression and the international infant growth charts by Fenton et al. as reference, the estimated decrease in birthweight for a 39-week male infant in response to a log₂ increment in maternal BLL exposure is 25 g. On the contrary, using quantile regression estimates, the corresponding decrease in birthweight for the same 39-week male infant would have been 50 g had the infant been in the 20th percentile, and 20 g had the infant been in the 80th percentile of the BWGA z-score distribution. Not only do our findings suggest that the magnitude of the association between prenatal exposure to lead at second trimester and birthweight-for-gestational age is higher among infants in the lower percentiles, but they also suggest that this association is significantly underestimated when linear regression is used. Knowing that infants in the lower BWGA z-score percentiles suffer the most, use of traditional linear regression methods may lead to underestimating the public health burden associated with the effects of lead on fetal growth.

When we examined the odds of SGA births across different quartiles of prenatal exposure to maternal BLL during second trimester, we observed a linear dose-response relationship. Our findings are in agreement with other studies with respect to the association
between prenatal exposure to lead and increased risk of SGA births; however, we found that this association occurs at much lower exposure levels than what has been previously reported. For example, in a study of 1,611 participants, Chen et al. found that mothers with BLL ≥20 μg/dL during pregnancy had an increased risk ratio (RR) of 2.15 (95% CI: 1.15-3.83) of SGA as compared to mothers with BLL <10 μg/dL (reference) (Chen et al. 2006). In another study with smaller sample size (n=262), Jelliffe-Pawlowski et al. found increased odds for SGA births of 4.2 (95% CI: 1.3-13.9) in women with BLL ≥10 μg/dL as compared to those with BLL <10 μg/dL (Jelliffe-Pawlowski et al. 2006). In our study, we found increased odds for SGA births of 1.62 (95% CI: 0.99-2.65) in women with BLL >4.53 μg/dL (Q4) as compared to those with <1.93 μg/dL (Q1). Our findings suggest that prenatal exposure to lead has a negative impact on the normal growth of the fetus even at lower concentrations than those previously reported. This is in agreement with the findings by Zhu et al. that suggested a no threshold effect of lead on birthweight (Zhu et al. 2010).

Our study has several limitations. The lack of ultrasound measurements and the use of the LMP method to estimate the gestational age at enrollment may have added some measurement error. In one of our previous work, we collected ultrasound data on 98 women and validated the LMP method, which yielded a high correlation (Spearman’s Rho: 0.9, p<0.0001) (Capurro et al. 1978). We also believe that any measurement error introduced by the use of LMP data collected in the 2nd trimester of pregnancy would be non-differential and most likely would drive effect estimates towards the null. We acknowledge that some residual measurement error from using the LMP data might still be present. The use of only second trimester maternal BLL data may not precisely reflect prenatal exposure throughout the course of pregnancy. Rabito et al. measured maternal BLL in the first, second and third trimester and found that second trimester is possibly the most critical window for the association between prenatal exposure to lead and birthweight (Rabito et al. 2014). Lastly, our findings should be
cautiously interpreted in the context of an observational study where the possibility of unmeasured residual confounding precludes causal inference.

Our study has several strengths including its prospective design, population size, and the detailed data collection. Furthermore, the use of BWGA z-score as calculated by the Fenton et al. method allows for comparison with other international cohorts. Last, we demonstrated for the first time the advantages of using quantile regression to determine the association of prenatal exposure to lead and birthweight. Quantile regression is a method that allows estimating the association independently of any outcome distribution. In contrast, linear regression makes assumptions about the distribution of the outcome and calculates the mean estimate across the whole outcome distribution. Our findings demonstrate that quantile regression is a useful tool to ascertain environmental effects which may vary at the tail ends of continuous outcomes, such as birthweight, cognitive test scores or BMI. When used together with linear regression, quantile regression can help to more precisely estimate the impact of environmental exposures on health outcomes and identify subpopulations that might be at highest risk.

CONCLUSIONS

Quantile regression revealed significant shifts in birthweight-for-gestational age distribution associated with prenatal exposure to lead at second trimester that were not fully captured by linear regression models. Our findings indicate that the magnitude of this association is larger and stronger in the lower percentiles of the birthweight-for-gestational age distribution. This provides evidence that infants with poorer fetal growth may represent a sensitive subpopulation for lead exposure. Improvements in maternal lead exposure early in pregnancy, may
significantly improve fetal growth, especially among infants who are at highest risk of long-term sequelae from poor fetal growth.
CONFLICT OF INTEREST

None declared.

AUTHORS’ CONTRIBUTIONS

RR and AAB developed the study concept. RR analyzed the data and wrote the manuscript. ROW and MMTR contributed participants’ material. CA measured lead in samples. HHB, KS, AMG, BC, MMTR and ROW provided critical revisions. AAB conceived the study, oversaw research, helped to write the manuscript, and provided intellectual input throughout the study. All authors have read and approved the final manuscript.

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Figure S2.1: Flow diagram of the study population.
CHAPTER 3

MicroRNAs in Serum Extracellular Vesicles May Predict Fetal Growth Early in Pregnancy

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*Corresponding author
ABSTRACT

Background

The placenta supports fetal development during the course of pregnancy, during which the number of extracellular vesicles (EVs) significantly increase in the maternal blood circulation. The abundance of microRNAs (miRNAs) in EVs (evmiRNAs) presents a unique opportunity for developing biomarkers for fetal growth.

Methods

We selected second trimester serum samples from 100 participants in the PROGRESS study. Samples were selected from pregnant women who delivered between 37-40 weeks of gestation. Women with infants that were small-for-gestational age (SGA) (n=36) and large-for-gestational age (LGA) (n=13) were matched to appropriate-for-gestational age (AGA) (n=51) by gestational age at delivery and infant’s sex. We also selected 40 samples across the birthweight-for-gestational age (BWGA) distribution. To screen for 754 evmiRNAs, we used the TaqMan OpenArray® Real-Time PCR platform. We compared the levels of evmiRNAs in pregnant women with infants that were SGA and LGA, versus AGA, using the fold change (FC) method. To determine the association between serum evmiRNAs and BWGA z-score, we used linear regression models, adjusting for maternal age, body mass index (BMI), blood lead levels, and parity.

Results

We detected 446 evmiRNAs in 100 analyzed serum samples, of which 126 were detected in ≥50% of the samples. We only considered these for further statistical analysis. While lower levels of miR-324-3p (FC=0.53, p=0.003), miR-20b-5p (FC=0.59, p=0.007), miR-942-5p (FC=0.49, p=0.007), miR-127-3p (FC=0.61, p=0.02), miR-25-3p (FC=0.66, p=0.04) and miR-21-
5p (FC=0.82, p=0.04) were detected in serum EVs from SGA versus AGA women, higher levels of miR-197-3p (FC=1.52, p=0.05) and miR-345-5p (FC=2.1, p=0.05) were detected in serum EVs from LGA versus AGA women. Last significant associations were found of mir-345-5p (β=-0.28, p=0.002), mir-10a-5p (β=-0.42, p=0.003), mir-1274B (β=-0.24, p=0.01), mir-127-3p (β=-0.34, p=0.01), mir-597-5p (β=-0.43, p=0.01), mir-483-5p (β=-0.19, p=0.02), mir-185-5p (β=-0.49, p=0.02), miR-197-3p (β=-0.33, p=0.05), miR-99b-3p (β=-0.19, p=0.05) and mir-139-5p (β=-0.22, p=0.05) with BWGA z-score.

**Conclusions**

Our study demonstrates the potential of evmiRNAs as early biomarkers of fetal growth. Further epidemiological studies are warranted to validate our findings and help establish a panel of easily quantifiable, highly sensitive and specific biomarkers for fetal growth during the course of pregnancy.

**Keywords:** Small-for-Gestational Age; Large-for-Gestational-Age; Birthweight; Exosomes; Microvesicles; C14MC; C19MC;
BACKGROUND

The placenta is a critical organ that develops during pregnancy to support fetal development and facilitate feto-maternal communication. During the course of pregnancy, the placenta transfers oxygen and nutrients from the mother to the fetus, filters out fetal waste excretions, and produces hormones, growth factors and cytokines that are essential for fetal development (Nelson 2015). Impairment of the placenta function not only affects fetal development (e.g., intrauterine growth restriction) (Thornburg et al. 2015), but also affects the mother’s physiology, which often leads to placenta-related complications such as maternal hypertension and pre-eclampsia (Redman et al. 2015). A healthy communication between mother, fetus and placenta during pregnancy is critical, but our knowledge of the mechanisms involved in this communication and how they affect maternal, fetal, and placental health, is very limited.

A recently characterized novel mechanism of cell-to-cell communication is extracellular vesicles (EVs) (Yanez-Mo et al. 2015), which are small double-lipid membrane vesicles (40-1000 nm in diameter) (Mathivanan et al. 2010) that encapsulate and transfer biological molecules such as proteins and nucleic acids from the cell of origin to the recipient cell (Arroyo et al. 2011; Skog et al. 2008; Vickers et al. 2011). Once EVs are up-taken by the recipient cell, their content (e.g., proteins and RNA molecules) can become functional and alter gene expression in the recipient cell (Valadi et al. 2007). MicroRNAs (miRNAs) are small non-coding RNA molecules (~22 nucleotides) (Bartel 2009) which are abundantly present in EVs (Mathivanan et al. 2010). Due to their complementary sequence to messenger RNA (mRNA) transcripts and binding affinity, miRNAs can potentially recognize, target, and prohibit translation of multiple mRNAs, either by initiating their degradation or by inhibiting the translational machinery. It has been estimated that miRNAs can post-transcriptionally regulate more than half of cellular gene expression (Bartel 2009).
The number of EVs in the circulating blood of pregnant women increases significantly compared to non-pregnant women (Salomon et al. 2014). New evidence has emerged that EVs and their cargo present an active mechanism of communication between mother, fetus, and the placenta. For example a few studies have shown that EVs released from the placenta into the maternal blood circulation can affect the inflammatory response of peripheral blood mononuclear cells (Holder et al. 2012; Southcombe et al. 2011), activate the complement (Biro et al. 2007), and carry specific miRNAs to recipient cells to modulate viral resistance (Delorme-Axford et al. 2013; Donker et al. 2012). RNA of fetal and placental origin can also be found in EVs in the maternal circulation (Ge et al. 2005; Go et al. 2004; Ng et al. 2003; Poon et al. 2000; Tsui et al. 2004; Williams et al. 2013). Several members of the placental-specific miRNA clusters on chromosomes 14 (C14MC) and 19 (C19MC) (Bentwich et al. 2005; Donker et al. 2012) that are implicated in placental regulation were found to be differentially expressed in abnormal placentas and detected in EVs circulating in maternal blood (Mouillet et al. 2015). A few studies have identified miRNAs that were differentially expressed in placentas from fetal growth restriction pregnancies (Higashijima et al. 2013; Hromadnikova et al. 2012; Mouillet et al. 2010); however, we have very limited evidence on the presence of evmiRNAs in maternal blood during pregnancy and their association with fetal growth.

We profiled and measured the levels of evmiRNAs in maternal serum, and determined their association with fetal growth by screening for the levels of 754 miRNAs in EVs isolated from second trimester serum samples of 100 pregnant women who participated in the Programming Research in Obesity, GRowth Environment and Social Stress (PROGRESS) prospective birth cohort study. We used serum samples from second trimester because we were interested in the development of early biomarkers for fetal growth and this was the earliest time point where samples were available. In this study, we hypothesized that miRNAs in EVs (evmiRNAs) circulating in maternal blood at second trimester can provide a unique and
minimally invasive approach to collect signals from the placenta and fetus that are informative for fetal growth.

**METHODS**

**Study design and sample selection**

Study participants were enrolled during their first prenatal care visit (<20 weeks of gestation) at the Mexican Social Security Institute (Instituto Mexicano del Seguro Social) clinics, as part of the PROGRESS prospective birth cohort study in Mexico City. The profile of the cohort is described elsewhere (Burris et al. 2013). Our base population was composed of pregnant women who agreed to participate (n=946) and had a live birth. The study was approved by the Institutional Review Boards of Brigham and Women’s Hospital, Harvard T.H. Chan School of Public Health, and the National Institute of Public Health in Mexico (#14265-101; #14706-101; #2006-P-001416; #2006-P-001792; #560). All study participants provided written informed consent in accordance with the Declaration of Helsinki of ethical principles for medical research.

Blood samples were collected in EDTA tubes (Becton Dickinson, Waltham, MA) at second trimester visit (12-24 weeks of gestation), and the serum fraction was separated by centrifugation at 1,500 x g for 15 minutes, following standard operating procedures. Serum was aliquoted in cryovial tubes, stored at -80°C, and shipped to the Harvard T.H. Chan School of Public Health, Laboratory of Environmental Epigenetics. Infants were considered as small-for-gestational age (SGA) if they were in the <10th percentile, appropriate-for-gestational age (AGA) if they were between 10th - 90th percentile, and large-for-gestational age (LGA) if they were in the >90th percentile of the BWGA distribution according to the international infant growth charts developed by Fenton et. al. (Fenton et al. 2013).
For our analysis, we selected 36 samples from women with SGA infants and 13 samples from women with LGA infants. We also selected 51 samples from women with AGA infants of which 36 were used as controls for SGA and 13 for LGA samples. AGA control samples were matched to SGA and LGA samples by gestational age at delivery and infant’s sex. The additional non-matched 19 AGA serum samples were randomly selected across the birthweight-for-gestational age (BWGA) distribution, totaling the number of 100 selected analyzed serum samples in our study. All serum samples were selected from pregnant women who delivered between 37 to 40 weeks of gestation. Gestational age at the time of enrollment was calculated using the date of last menstrual period (LMP) and by an additional standardized physical examination assessment at birth (Capurro et al. 1978).

**RNA extraction and miRNA profiling**

Serum samples were thawed on ice and filtered using a 0.8μm membrane unit (Millipore Corp., Bedford, MA) to remove any cell debris and large aggregates. Following this step, we used the exoRNeasy Serum/Plasma Maxi kit (Qiagen, Valencia, CA) to extract total RNA from EVs from ~700μL of serum following the manufacturer’s protocol (Enderle et al. 2015). Total RNA was eluted in ~12μL volumes and subsequently concentrated to 6μL volumes using the RNA Clean & Concentrator™-5 (Zymo, Irvine, CA), prior to miRNA analysis. We used the TaqMan OpenArray® technology (Life Technologies, Carlsbad, CA) to measure the expression profile of 754 miRNAs (miRBase v14).

All RNA samples were reverse transcribed and pre-amplified (16 cycles) according to the manufacturer's protocol using Megaplex™ Reverse Transcription Primers, Human Pool A v2.1, Human Pool B v3.0 and Megaplex™ PreAmp Primers, Human Pool A v2.1 and Human Pool B v3.0. The quantitative polymerase chain reaction (qPCR) analysis was performed on the QuantStudio™ 12K Flex Real-Time PCR System (Life Technologies, Carlsbad, CA) and relative
threshold cycle (C_r) values were calculated. C_r values were defined as the PCR cycle where the amplification curve exceeded the background fluorescence threshold line.

**Data analysis**

We used standard statistics to quantify the study participants’ characteristics. Data are reported as mean ± standard deviation (SD), unless otherwise specified. We performed quality control on raw qPCR miRNA data according to the following inclusion criteria: (a) C_r values <35, (b) amplification scores ≥1.1, and (c) C_q confidence ≥0.8 ((Applied Biosystems) 2012). Data that did not meet these criteria were treated as missing values in the analysis. Following this step, evmiRNAs were ranked based on their frequency of detection across all analyzed samples, and only evmiRNAs detected in ≥50% of the analyzed samples were considered for further statistical analysis.

We used the global mean method to normalize our data, and calculated Delta C_r (ΔC_r) values according to the ΔC_r,miRNAi = (C_r,miRNAi - C_r,miRNAi_global_mean) formula, as described by Mestdagh et al. (Mestdagh et al. 2009). To compare the evmiRNA profile in pregnant women with SGA, AGA, and LGA infants (SGA versus AGA, and LGA versus AGA), we used the fold change method according to the 2^ΔΔCt formula (Livak et al. 2001). The Student’s paired t-test was performed to calculate statistical significance of the fold change differences between compared groups.

We used linear regression models to determine the association between the levels of serum evmiRNAs at second trimester and BWGA z-score. All models were adjusted for maternal age (continuous), body mass index (BMI) (continuous), blood lead levels (continuous), parity (multiparous or nulliparous), and infant’s sex. Last, to evaluate the prediction value of detected evmiRNAs, we used logistic regression models to construct Receiver Operating Characteristic (ROC) curves. The area under the curve (AUC), R^2, sensitivity, specificity, and
negative and positive predictive values were calculated for each evmiRNA. All statistical analyses were performed in SAS version 9.4 (SAS Institute Inc., Cary, NC).

RESULTS

Characteristics of study participants

We found that women with LGA infants were older than those with SGA infants (p=0.03), and had higher BMI (p=0.003) than women with both SGA and AGA infants (Table 3.1). The mean ± SD second trimester maternal blood lead level was slightly higher, but not statistically significant, in the SGA group (4.4 ± 3.2 μg/dL) as compared to the AGA (3.5 ± 3.5 μg/dL) and LGA (3.7 ± 2.4 μg/dL) groups. No differences were observed in any other characteristics of the study participants between the SGA, AGA, and LGA groups.
| Characteristics                      | All Participants | Matched groups | Matched groups | Matched groups | Matched groups | Matched groups | Matched groups | Matched groups | P value<sup>d</sup> |
|-------------------------------------|------------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|
| N                                  | 100              | 36             | 51             | 13             |                |                |                |                |                | 0.03           |
| **Maternal**                        |                  |                |                |                |                |                |                |                |                |                |
| Age (years)                         | 27.8 ± 6.1       | 26.0 ± 6.1     | 28.4 ± 5.8     | 30.6 ± 6.4     | 0.03           |
| Height (cm)                         | 154.4 ± 5.1      | 153.4 ± 5.4    | 154.7 ± 4.8    | 156.0 ± 4.9    | 0.09           |
| Body weight (kg)                    | 62.7 ± 9.5       | 61.0 ± 10.8    | 61.8 ± 7.9     | 71.2 ± 7.6     | 0.001          |
| Body mass index (BMI) (kg/m²)       | 26.3 ± 3.7       | 25.9 ± 4.1     | 25.8 ± 3.3     | 29.2 ± 2.6     | 0.003          |
| Hemoglobin levels (g/dL)            | 12.8 ± 1.0       | 13.0 ± 1.1     | 12.7 ± 0.9     | 12.7 ± 1.4     | 0.44           |
| Blood lead concentration (µg/dL)    | 3.9 ± 2.8        | 4.4 ± 3.2      | 3.5 ± 2.5      | 3.7 ± 2.4      | 0.33           |
| Multiparous; n (%)                  | 65 (65.0)        | 20 (55.6)      | 34 (66.7)      | 11 (84.6)      | 0.17           |
| Alcohol consumption; n (%)          |                  |                |                |                | 0.72           |
| Yes                                 | 6 (6.0)          | 3 (8.3)        | 3 (5.9)        | 0 (0.0)        |                |
| No                                  | 94 (94.0)        | 33 (91.7)      | 48 (94.1)      | 13 (100.0)     |                |
| Socioeconomic status (SES); n (%)   |                  |                |                |                | 0.93           |
| Low                                 | 49 (49.0)        | 17 (47.2)      | 25 (49.0)      | 7 (53.8)       |                |
| Medium                              | 40 (40.0)        | 15 (41.7)      | 21 (41.2)      | 4 (30.8)       |                |
| High                                | 11 (11.0)        | 4 (11.1)       | 5 (9.8)        | 2 (15.4)       |                |
| Smoke exposure in the home<sup>e</sup>; n (%) |         |                |                |                | 0.49           |
| Yes                                 | 28 (28.6)        | 9 (25.7)       | 17 (33.3)      | 2 (16.7)       |                |
| No                                  | 70 (71.4)        | 26 (74.3)      | 34 (66.7)      | 10 (83.3)      |                |
| **Infant**                          |                  |                |                |                |                |                |                |                |                |
| Birth weight (g)                    | 3014 ± 633       | 2396 ± 220     | 3190 ± 393     | 4038 ± 331     | <0.0001        |
| Gestational age (weeks)             | 38.3 ± 1.0       | 38.2 ± 1.0     | 38.3 ± 1.1     | 38.3 ± 1.0     | 0.89           |
| Birthweight-for-gestational age<sup>f</sup> (z-score) | -0.6 ± 1.3 | -1.9 ± 0.4 | -0.2 ± 0.7 | 1.5 ± 0.3 | <0.0001 |
| Sex; n (%)                          |                  |                |                |                | 1.00           |
| Males                               | 51 (51.0)        | 18 (50.0)      | 26 (51.0)      | 7 (53.8)       |                |
| Females                             | 49 (49.0)        | 18 (50.0)      | 25 (49.0)      | 6 (46.2)       |                |

<sup>a</sup>All maternal information was collected during the second trimester visit (12-24 weeks).

<sup>b</sup>All values are shown as mean ± SD, unless otherwise noted.

<sup>c</sup>Participants in the SGA, AGA, and LGA groups were matched by gestational age at delivery and infant's sex.

<sup>d</sup>P value corresponds to Chi squared (continuous) or Fisher's exact (categorical) test for the difference in study participants' characteristics between SGA, AGA, and LGA groups.

<sup>e</sup>Data are missing for 2 participants.

<sup>f</sup>Birthweight is adjusted for gestational age, and z-score is calculated based on the Fenton growth charts.
Levels of evmiRNAs in second trimester maternal serum and fetal growth

A total of 446 evmiRNAs were detected in at least one of the analyzed serum samples (n=100), but only 126 evmiRNAs were detected in ≥50% of the analyzed samples. The profile of all 126 evmiRNA as ranked by frequency of detection and Ct levels is shown in Figure 3.1. We found that 50 evmiRNAs were detected in ≥90% of the analyzed samples, including highly abundant evmiRNAs such as miR-150-5p, miR-17-5p, miR-191-5p, miR-223-3p, miR-1274B, miR-24-3p and miR-30c-5p (Figure 3.1; Table S3.1).

Significant fold change differences were found in the profile of evmiRNAs detected in women with SGA, AGA and LGA infants (Figure 3.2). In particular, significantly lower levels (fold change) were found for miR-324-3p (0.53, p=0.003), miR-20b-5p (0.59, p=0.007), miR-942-5p (0.49, p=0.007), miR-127-3p (0.61, p=0.02), miR-25-3p (0.66, p=0.04) and miR-21-5p (0.82, p=0.04) in serum samples from women with SGA as compared to AGA infants. Significantly higher levels were found for miR-197-3p (1.52, p=0.05) and miR-345-5p (2.1, p=0.05) in serum samples from women with LGA versus AGA infants. The full list of evmiRNAs in association with SGA and LGA is provided in Table S3.2 and Table S3.3.

When we tested for the association between levels of evmiRNAs and BWGA z-scores, significant associations were found that lower levels of mir-345-5p (-0.28, p=0.002), mir-10a-5p (-0.42, p=0.003), mir-1274B (-0.24, p=0.01), mir-127-3p (-0.34, p=0.01), mir-597-5p (-0.43, p=0.01), mir-483-5p (-0.19, p=0.02), mir-185-5p (-0.49, p=0.02), mirR-197-3p (-0.33, p=0.05), mirR-99b-3p (-0.19, p=0.05) and mir-139-5p (-0.22, p=0.05) were associated with lower BWGA z-score (Figure 3.3; Table S3.4).
Figure 3.1: Heat map showing miRNAs in serum extracellular vesicles (evmiRNAs) detected in ≥50% of the analyzed samples.

The profile of 126 evmiRNAs that were detected in ≥50% of the analyzed samples (n=100) is summarized in this heat map. All evmiRNAs were ranked by frequency (high to low) and levels of detection (high to low) as measured by cycle threshold (C_r) values. The most frequently detected and highly expressed evmiRNAs are shown at the top of the heat map. Expression levels are shown in C_r values; red color indicates higher and yellow color indicates lower expression levels; white color indicates that a specific evmiRNA was not detected in this sample.
Figure 3.2: Fold change of miRNAs detected in serum extracellular vesicles (evmiRNAs) between mothers with small-, appropriate, and large-for-gestational age infants.

Volcano plot showing the fold change of all tested serum evmiRNAs from second trimester of pregnancy of mothers with SGA versus AGA infants (n=36 pairs) (white) and LGA versus AGA (n=13 pairs) (grey). Samples in the groups were matched by gestational age at delivery and infant’s sex.
Figure 3.3: Associations between individual miRNAs detected in serum extracellular vesicles (evmiRNAs) and birthweight-for-gestational age z-score (n=100).

Volcano plot showing the association between individual miRNAs in serum evmiRNAs at second trimester and birthweight-for-gestational age z-score; estimates are adjusted for maternal age, body mass index, blood lead levels, parity, and infant’s sex; $-\log_{10}(P\ Value)$ indicates transformed p-values of the association between each evmiRNA and birthweight-for-gestational age z-score.
Predictive potential of second trimester evmiRNAs to detect SGA and LGA infants

We used ROC curves to evaluate the predictive potential of evmiRNAs to detect SGA and LGA pregnancies in the second trimester. We found that miR-942-5p exhibited the highest potential as an early biomarker for SGA with an AUC of 0.78 (p=0.02) and $R^2$ of 0.18. Other evmiRNAs that showed biomarker potential were miR-324-3p with an AUC of 0.74 (p=0.02) and $R^2$ of 0.17, miR-20b-5p with an AUC of 0.70 (p=0.02) and $R^2$ of 0.12, and miR-127-3p with an AUC of 0.71 (p=0.09) and $R^2$ of 0.09. The highest sensitivity for SGA was achieved by miR-324-3p (0.86) and the highest specificity was achieved by miR-942-5p (0.86) (Table 3.2).

Similarly, evmiRNAs that showed the highest potential as early biomarkers for LGA were miR-197-3p with an AUC of 0.79 (p=0.05) and $R^2$ of 0.22, miR-10a-5p with an AUC of 0.72 (p=0.08) and $R^2$ of 0.17, miR-345-5p with an AUC of 0.69 (p=0.10) and $R^2$ of 0.12, and miR-222-3p with an AUC of 0.69 (p=0.11) and $R^2$ of 0.15. The highest sensitivity was achieved miR-10a-5p and miR-345-5p (1.00); whereas the highest specificity was achieved by miR-197-3p (0.82) (Table 3.3).

Table 3.2: Predictive potential of evmiRNAs in detecting small-for-gestational age infants at second trimester.

<table>
<thead>
<tr>
<th>miRNA</th>
<th>AUC</th>
<th>$R^2$</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>NPV</th>
<th>PPV</th>
<th>P value</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-942-5p</td>
<td>0.78</td>
<td>0.18</td>
<td>0.70</td>
<td>0.86</td>
<td>0.75</td>
<td>0.82</td>
<td>0.02</td>
<td>41</td>
</tr>
<tr>
<td>miR-324-3p</td>
<td>0.74</td>
<td>0.17</td>
<td>0.86</td>
<td>0.61</td>
<td>0.79</td>
<td>0.72</td>
<td>0.02</td>
<td>39</td>
</tr>
<tr>
<td>miR-20b-5p</td>
<td>0.70</td>
<td>0.12</td>
<td>0.59</td>
<td>0.78</td>
<td>0.62</td>
<td>0.76</td>
<td>0.02</td>
<td>59</td>
</tr>
<tr>
<td>miR-127-3p</td>
<td>0.71</td>
<td>0.09</td>
<td>0.60</td>
<td>0.81</td>
<td>0.83</td>
<td>0.80</td>
<td>0.09</td>
<td>36</td>
</tr>
</tbody>
</table>
DISCUSSION

In this study we conducted a high-throughput screening of the levels of 754 miRNAs in extracellular vesicles from 100 serum samples collected from pregnant women in the second trimester. We detected the presence of 446 evmiRNAs, of which 126 were detected in ≥50% of the analyzed samples. We also observed a pattern of overall lower evmiRNA levels in second trimester serum samples of pregnant women who delivered SGA infants, and higher evmiRNA levels in those who delivered LGA infants, when compared to those with AGA infants. In particular, we found lower levels of miR-324-3p, miR-20b-5p, miR-942-5p, miR-127-3p (0.61, p=0.02), miR-25-3p (0.66, p=0.04) and miR-21-5p (0.82, p=0.04) in serum samples of pregnant women who delivered SGA versus AGA infants. We also identified several evmiRNAs that had higher levels in serum samples of pregnant women who delivered LGA versus AGA infants, with miR-197-3p and miR-345-5p being the most notable. We identified nine evmiRNAs (i.e., mir-345-5p, mir-10a-5p, mir-1274B, mir-127-3p, mir-597-5p, mir-483-5p, mir-185-5p, mir-197-3p, mir-99b-3p and mir-139-5p) that were significantly associated with birthweight-for-gestational age z-score. To the best of our knowledge, several of these evmiRNAs have not previously been associated with fetal growth. Taken together, these findings are consistent with our hypothesis that evmiRNAs detected in maternal blood circulation as early as the second trimester have predictive potential for detecting large-for-gestational age infants at second trimester.

Table 3.3: Predictive potential of evmiRNAs in detecting large-for-gestational age infants at second trimester.

<table>
<thead>
<tr>
<th>miRNA</th>
<th>AUC</th>
<th>R²</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>NPV</th>
<th>PPV</th>
<th>P value</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-197-3p</td>
<td>0.79</td>
<td>0.22</td>
<td>0.91</td>
<td>0.82</td>
<td>0.90</td>
<td>0.83</td>
<td>0.05</td>
<td>22</td>
</tr>
<tr>
<td>miR-10a-5p</td>
<td>0.72</td>
<td>0.17</td>
<td>1.00</td>
<td>0.56</td>
<td>1.00</td>
<td>0.73</td>
<td>0.08</td>
<td>20</td>
</tr>
<tr>
<td>miR-345-5p</td>
<td>0.69</td>
<td>0.12</td>
<td>1.00</td>
<td>0.42</td>
<td>1.00</td>
<td>0.63</td>
<td>0.10</td>
<td>24</td>
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<tr>
<td>miR-222-3p</td>
<td>0.69</td>
<td>0.15</td>
<td>0.70</td>
<td>0.73</td>
<td>0.73</td>
<td>0.70</td>
<td>0.11</td>
<td>21</td>
</tr>
</tbody>
</table>
trimester of pregnancy can provide valuable and easily accessible information about fetal
growth.

Previous studies have identified several miRNAs that were differentially expressed in
placentas from complicated pregnancies, suggesting their involvement in the regulation of
placental and fetal development and emerging evidence shows that several of these miRNAs
can be detected in EVs that circulate in maternal blood during pregnancy. Many of these studies
focused on miRNAs of the placenta-specific C14MC (e.g., miR-376c-3p) and C19MC (e.g., miR-
517a-3p, miR-520h and miR-525-3p) clusters. These were found to not only be involved in
preeclampsia (Manokhina et al. 2015), but in other pregnancy-related complications as well,
including impaired fetal growth (Hu et al. 2014; Jiang et al. 2015). In our study, we found no
significant associations with any of the detected C19MC miRNA members and fetal growth. Our
findings are consistent with the findings by Hromadnikova et. al., in which they investigated the
levels of 7 C19MC placenta-specific miRNAs (i.e., miR-516-5p, miR-517-5p, miR-518b, miR-
520a-5p, miR-520h, miR-525-3p, and miR-526a-3p) in the plasma of fetal growth restriction and
normal pregnancies and found no significant differences (Hromadnikova et al. 2012). In a similar
study, Mouillet et al. compared the levels of 12 placenta-specific miRNAs, including members of
the C19MC cluster (i.e., miR-517-5p, miR-518b, miR-518e and miR-524), in plasma samples
from gestational age-matched normal and fetal growth restricted pregnancies (Mouillet et al.
2010); the investigators reported no significant differences in any of the screened C19MC
miRNAs. One study by Fu et al., found that miR-376c-3p, which is a member of the C14MC
cluster, was implicated in preeclampsia (Fu et al. 2013); however, we did not find any significant
association of miR-376c-3p and fetal growth in our study. However, we did find a significant
association with another member of the C14MC cluster, miR-127-3p, which had not been
evaluated in relation to fetal growth by previous studies.
Our finding that miR-127-3p was among the most significantly associated evmiRNAs with fetal growth is intriguing. Significantly lower levels of miR-127-3p were detected in serum EVs of mothers who delivered SGA infants and higher but not significantly different levels were detected in serum EVs of mothers who delivered LGA infants, compared to those with AGA infants. When we tested for the association of miR-127-3p and BWGA z-score using data from all 100 analyzed samples, we found a significant positive association. This finding suggests a dose-response relationship between miR-127-3p and fetal growth. Interestingly, miR-127-3p is a member of the C14MC miRNA cluster, which is known to be highly expressed in the placenta; however, our knowledge of the role of this cluster in placental function is still very limited. A study by Labialle et al., showed that while knockout of the C14MC cluster in mice was not associated with specific placental adverse phenotypes, these mice exhibited partially penetrant neonatal lethality (Labialle et al. 2014). A more recent study by Ito et al. showed that when miR-127 was deleted in mice, insufficiencies in the labyrinthine zone of the placenta were developed, which is analogous to the human placenta villous area that is directly involved in the transfer of gases and nutrients between the mother and the fetus (Ito et al. 2015). These findings suggest that miR-127-3p is implicated in placental development and fetal growth.

We also found significant associations between fetal growth and several members of the miR-17~92 cluster, which is expressed in a wide spectrum of tissues (Mogilyansky et al. 2013) and that has been suggested to play an important role in normal embryonic development (Houbaviy et al. 2003; Ventura et al. 2008). Members of this cluster were previously found to be associated with pregnancy complications, in particular preeclampsia, suggesting their importance in healthy pregnancies and normal placental and fetal development (Manokhina et al. 2015). In our study, we found that levels of miR-20b-5p were significantly lower between participants with SGA, as compared to those with AGA infants. Wang et al. found that expression of miR-20b-5p was altered in placentas from women with pre-eclampsia, suggesting
its regulating potential in placental angiogenesis and trophoblast development (Wang et al. 2012), processes that are important in normal fetal development. We also found that the levels of miR-25-3p were detected in lower levels in EVs from participants with SGA versus AGA infants. Increased levels of miR-25-3p were found to be associated with fetal macrosomia in at least two other studies, indicating its possible role in normal fetal growth (Ge et al. 2015; Jiang et al. 2015).

One of the most significantly associated evmiRNAs with SGA was miR-942-5p, which is implicated in angiogenesis. Although normal angiogenesis is an important process for placental development, two studies that examined the association of miR-942-5p and preeclampsia found no significant results (Hromadnikova et al. 2014; Luque et al. 2014). In addition, we found 8 evmiRNAs (miR-10a-5p, miR-1274B, miR-139-5p, miR-197-3p, miR-25-3p, miR-483-5p, miR-597-5p and miR-99b-3p) that were significantly associated with fetal growth in our study but had not been reported by other studies.

Our study has several limitations. We profiled miRNAs extracted from the pool of EVs circulating in maternal blood and those originate from different cells/tissues that communicate with blood including blood cells, the liver, and of course the placenta. We therefore cannot identify the source of origin of the miRNAs that were found to be of interest. However, the fact that some of the miRNAs are only expressed, or highly expressed in the placenta (e.g. C14MC and C19MC members) and were detected in EVs in our study, provides some evidence for their placental origin. In addition, our findings have not been validated that may preclude generalizability to other populations. We also want to mention that, despite the large number of 754 miRNAs we screened for, we cannot exclude the possibility that other known miRNAs that were not included in this study might be associated with fetal growth. Last, we want to acknowledge that the relatively small sample size in our study might have limited our statistical
power in the associations we observed between fetal growth and evmiRNAs, as well as our ability to detect more subtle associations.

One of the advantages of our study was the nested case-control design, with careful matching by gestational age at delivery and infant’s sex. We also evaluated several candidate confounders of the association between evmiRNAs levels and fetal growth, such as maternal age, BMI, SES index, hemoglobin levels and parity, which provided an optimal design to capture true differences in evmiRNAs that might be associated with fetal growth. In addition, the homogeneity of the study population helped reduce any unmeasured confounding that might be attributed to differences in population genetics.

**CONCLUSIONS**

This study demonstrates the potential of several evmiRNAs many of which are placenta specific, as early biomarkers of fetal growth. Further epidemiological studies are warranted to validate our findings in other populations. Our findings provide evidence on the unique opportunity we have to develop minimally invasive biomarkers for fetal growth which could be used to identify, monitor, and evaluate high risk subpopulations throughout the course of pregnancy.
CONFLICT OF INTEREST
None declared.

AUTHORS’ CONTRIBUTIONS
RR and AAB developed the study concept. RR designed and performed the experiments, analyzed the data, and wrote the manuscript. ZZ and JS helped in data analysis. ROW and MMTR contributed participants' material. ROW, MMTR and BC provided critical revisions. AAB oversaw research, helped to write the manuscript, and provided intellectual input throughout the study. All authors have read and approved the final manuscript.

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ACKNOWLEDGMENTS
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REFERENCES

https://tools.thermofisher.com/content/sfs/manuals/cms_101435.pdf


### Table S3.1: Detected miRNAs in serum extracellular vesicles (evmiRNAs).

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</tr>
</tbody>
</table>

*Data are shown in Crt (Relative Cycle Threshold) values.
†Number of samples (out of 100 analyzed) each miRNA was detected.
Table S3.2: Significantly dysregulated serum evmiRNAs at second trimester between women with SGA and AGA infants (n=36 pairs).

<table>
<thead>
<tr>
<th>miRNA</th>
<th>Fold Change</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Down-regulated</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>hsa-miR-324-3p</td>
<td>0.53</td>
<td>0.003</td>
</tr>
<tr>
<td>hsa-miR-20b-5p*</td>
<td>0.59</td>
<td>0.007</td>
</tr>
<tr>
<td>hsa-miR-942-5p</td>
<td>0.49</td>
<td>0.007</td>
</tr>
<tr>
<td>hsa-miR-127-3p†</td>
<td>0.61</td>
<td>0.02</td>
</tr>
<tr>
<td>hsa-miR-483-5p</td>
<td>0.73</td>
<td>0.02</td>
</tr>
<tr>
<td>hsa-miR-25-3p*</td>
<td>0.66</td>
<td>0.04</td>
</tr>
<tr>
<td>hsa-miR-21-5p</td>
<td>0.82</td>
<td>0.04</td>
</tr>
<tr>
<td>hsa-miR-223-5p</td>
<td>0.66</td>
<td>0.06</td>
</tr>
<tr>
<td>hsa-miR-148b-3p</td>
<td>0.83</td>
<td>0.06</td>
</tr>
<tr>
<td>hsa-miR-345-5p</td>
<td>0.67</td>
<td>0.09</td>
</tr>
<tr>
<td>hsa-miR-139-5p</td>
<td>0.77</td>
<td>0.09</td>
</tr>
<tr>
<td>hsa-miR-518d-3p§</td>
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<td>0.10</td>
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<tr>
<td><strong>Up-regulated</strong></td>
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<tr>
<td>hsa-miR-206</td>
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</tr>
<tr>
<td>hsa-miR-215-5p</td>
<td>1.18</td>
<td>0.09</td>
</tr>
</tbody>
</table>

*Member of the 17~92 miRNA cluster.
†Member of the C14MC miRNA cluster.
§Member of the C19MC miRNA cluster.

Table S3.3: Significantly dysregulated serum evmiRNAs at second trimester between women with LGA and AGA infants (n=13 pairs).

<table>
<thead>
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<th>Fold Change</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Up-regulated</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>hsa-miR-197-3p</td>
<td>1.52</td>
<td>0.05</td>
</tr>
<tr>
<td>hsa-miR-345-5p</td>
<td>2.12</td>
<td>0.05</td>
</tr>
<tr>
<td>hsa-miR-222-3p</td>
<td>3.78</td>
<td>0.08</td>
</tr>
<tr>
<td>hsa-miR-638</td>
<td>1.20</td>
<td>0.08</td>
</tr>
<tr>
<td>hsa-miR-215-5p</td>
<td>1.56</td>
<td>0.09</td>
</tr>
<tr>
<td>hsa-miR-10a-5p</td>
<td>1.46</td>
<td>0.09</td>
</tr>
</tbody>
</table>
Table S3.4: Associations between miRNAs detected in extracellular vesicles and birthweight-for-gestational age z-score.

<table>
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<tr>
<th>miRNA</th>
<th>Difference in z-score</th>
<th>SE</th>
<th>P Value</th>
<th>-log_{10}(P Value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsa-miR-345-5p</td>
<td>-0.28</td>
<td>0.09</td>
<td>0.002</td>
<td>2.62</td>
</tr>
<tr>
<td>hsa-miR-10a-5p</td>
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<td>0.13</td>
<td>0.003</td>
<td>2.59</td>
</tr>
<tr>
<td>hsa-miR-1274B</td>
<td>-0.24</td>
<td>0.09</td>
<td>0.01</td>
<td>2.18</td>
</tr>
<tr>
<td>hsa-miR-127-3p</td>
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<td>0.12</td>
<td>0.01</td>
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</tr>
<tr>
<td>hsa-miR-597-5p</td>
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<td>0.01</td>
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</tr>
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<td>hsa-miR-483-5</td>
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<td>0.16</td>
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<td>1.32</td>
</tr>
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<td>0.05</td>
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(Table S3.4 Continued)

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<th>Log10 FDR</th>
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<tr>
<td>hsa-miR-376a-3p</td>
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*Adjusted for maternal age, body mass index, blood lead levels, parity and infant's sex.
SUMMARY AND CONCLUSIONS

The goal of this dissertation was to determine the potential of miRNAs encapsulated in extracellular vesicles as easily-accessible biomarkers to help us better understand in real time key underlying mechanisms that are involved different stages of disease, as well as to identify high risk individuals in relation to certain environmental exposures. In particular, we sought to address this question within the framework of two major public health problems: (a) air pollution and cardiovascular disease, and (b) prenatal lead exposure and fetal growth. The findings of this work were extremely promising and highlighted the usefulness of evmiRNAs in better understanding the underlying biological mechanisms involved in the onset of adverse health outcomes and how they respond to toxic environmental exposures.

The results of Chapter One indicated that long-term exposure to ambient PM$_{2.5}$ is associated with increased levels of several evmiRNAs circulating in the blood of older individuals. Further investigation of the biological relevance of PM$_{2.5}$-induced evmiRNAs revealed their involvement in CVD-related pathways, such as atherosclerosis, cardiac hypertrophy and cytokines mediating the communication between immune cells. Collectively, the findings of this chapter suggest that levels of evmiRNAs may reflect exposures to air pollution and may participate in biological functions relevant to CVD-related pathways.

Chapter Two demonstrated a significant negative association between prenatal lead exposure and fetal growth. It also highlighted that infants in the lower percentiles of the birthweight-for-gestational age (BWGA) distribution might experience greater magnitudes of the effect of lead on BWGA. This observation became apparent only after we employed quantile regression models, in addition to traditional linear regression models. This finding is very important, and it suggests that effects of prenatal lead exposure on birthweight would have been masked had linear regression models only been used.
In an extension of Chapter’s Two work, in Chapter Three we determined the value of evmiRNAs as early biomarkers of fetal growth, including several miRNAs that are specifically expressed in the placenta. In particular, findings of this chapter highlighted the potential of specific evmiRNAs, measured in the serum of pregnant women, as biomarkers of fetal growth as early as the second trimester of pregnancy. It also demonstrated the prognostic potential of certain evmiRNAs for small-, appropriate-, and large-for-gestational age infants early in pregnancy. evmiRNAs that were identified as candidate biomarkers of fetal growth in this chapter were usually or placental origin or involved in the regulation of key biological pathway related to placental and fetal development.

It is important to mention that the findings of this work should be very carefully interpreted within the context of observational studies. Also, given the relatively small number of samples we used to screen for evmiRNAs, as well as the homogeneity of the study participants, definitive and generalizable conclusions cannot be made. In addition, the limited amount of sample, a common problem in epidemiological studies, did not allow us to measure additional characteristics of EVs (e.g., number of EVs and surface markers), something that could have added important information in our study. Despite the abovementioned limitations, this work has several notable strengths as well. The utilization of samples which were prospectively collected, together with other information about the study participants, allowed us to very carefully develop our statistical models, adjust for several confounders, and obtain unbiased estimates to the extent possible. In addition, the use of high-throughput techniques to screen for a large number of evmiRNAs allowed us to measure several evmiRNAs that were not previously evaluated in relation to cardiovascular disease or fetal growth. Therefore, we believe that our work provides evidence to encourage further investigation by others to replicate and build on our findings in larger and more diverse populations.
Taken together, the work presented in this dissertation suggests that the use of EVs in the field of Environmental Epidemiology is very promising, and provides evidence that evmiRNAs can become an extremely useful and easily accessible source of biological information. Given the lack of non-invasive or minimally invasive ways to study tissues such as the heart or the placenta in real time, we believe that leveraging EVs and their content will become very critical in successfully addressing important public health questions in the future. We hope that this work contributed valuable information to the scientific community leading the way forward to healthier life spans.