Mixed Metals Exposure, Cardiac Autonomic Responses, Inflammation and DNA Methylation

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MIXED METALS EXPOSURE, CARDIAC AUTONOMIC RESPONSES, INFLAMMATION AND DNA METHYLATION

Jinming Zhang

A Dissertation Submitted to the Faculty of

The Harvard T. Chan School of Public Health

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for the Degree of Doctor of Science

in the Department of Environmental Health

Harvard University

Boston, Massachusetts.

May, 2016
Dissertation Advisor: Dr. David C. Christiani

Jinming Zhang

Mixed Metals exposure, cardiac autonomic responses, inflammation and DNA methylation

Abstract

Background: welders are often exposed to various types of metals from the welding fumes and they also have high risks of cardiac autonomic dysfunction. Knowing the hazardous components within metals mixture as well as understanding the potential underlying mechanisms is essential for environmental and occupational regulations. Objective: to identify metal components which are associated with cardiac autonomic responses, as measured by two novel markers - acceleration capacity (AC) and deceleration capacity (DC); to examine whether inflammation mediates effects of metals exposure on AC/DC changes; to identify epigenetic variants which are associated with AC/DC changes. Methods: we collected urine, blood and electrocardiogram (ECG) samples from 75 welders over six sampling occasions between June 2003 and June 2012. Urinary concentrations of 16 types of metals were determined. Blood serum samples were analyzed for inflammatory cytokines levels including CRP, IL-2, IL4, IL6 and IL8. AC and DC values were quantified from ECG recordings. Firstly, we used linear mixed-effects models with Lasso to identify hazardous metals that were significantly associated with AC or DC changes. We fitted the co-pollutants model with “selected” metals in the linear mixed model to estimate the exposure-response relationship. Then, we conducted a mediation analysis to examine whether inflammatory cytokines mediated the effects of metals exposure on AC or DC changes. We report both direct and indirect effects in single pollutant model as well as co-pollutants models. Finally, we conducted the epigenome-wide association study (EWAS) to identify epigenetic variants that were associated with AC or DC changes. Results: we observed negative associations between urinary mercury and chromium concentrations with both DC and AC changes. Indirect effects of metals exposure on AC or DC through inflammation pathway were not significant. We identified GPR133 gene at which methylation level changes were associated with DC values. Conclusion: metals exposures are associated with impaired cardiac
autonomic functions. Our study did not provide evidence that these effects were mediated through inflammation pathway. However, DNA methylation of specific genes may be a potential pathway linking environmental and occupational pollutants exposure and alterations in cardiac autonomic responses.
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Finally, I deeply thank my parents for giving me life, love and care, my older sister for all the support and being a friend who would listen to me all the time and all my friends for their help and encouragement all the way.

Jinming Zhang

May 2016
CHAPTER 1. INTRODUCTION
Over the last few decades, many advances have occurred in identifying roles of various types of environmental and occupational pollutants in adverse cardiovascular effects. Fine particulate matters (PM2.5) [1-2] or transitional metals [3] exposures have been suggested to pose increased risks of cardiovascular diseases partially through affecting the cardiac autonomic functions. In the heart, cardiac electrophysiology is modulated primarily by the cardiac autonomic nervous system, which consists of two major branches - the sympathetic system, associated with increased conduction velocity and heart rate, and the parasympathetic system, associated with restorative functions and decreased heart rate [4-5].

Under resting conditions, these branches are in dynamic balance. Nonetheless, environmental hazardous factors may trigger imbalance between the two branches. Typically, excessive energy demands occur when sympathetic branch dominates over the parasympathetic branch and when this effect lasts for long periods of time, the energy demands on the system cannot be met and death may occur eventually [6]. Both toxicological and epidemiological studies have documented evidence that autonomic imbalance have been associated with various pathophysiological conditions, such as diabetes [7-8] and hypertension [9-10].

Conventional hear rate variability (HRV) parameters, such as SDNN (the standard deviation of NN intervals), rMSSD (root mean square of successive differences), LF (low frequency) and HF (high frequency) have been extensively used as measurements of cardiac autonomic responses in previous research. However, more recent research suggests that those parameters may be neither accurate nor precise, as their biological mechanisms are unclear [11]. In addition, those parameters fail to account for heart rate and confounding bias may be introduced [12-13]. Bauer et al. proposed a new method named the phase-rectified signal averaging (PRSA) to separately quantify “acceleration capacity” (AC) and “deceleration capacity” (DC) of heart rate. Compared with conventional HRV parameter as well as left ventricular ejection fraction (LVEF), decreased DC has been suggested as a more precise predictor of cardiovascular morbidity and mortality in studies among patients after myocardial infarction [12,14].
Although the underlying biological mechanisms of alterations in cardiac autonomic responses following metals exposure are not known, several studies have suggested that inflammatory responses might play a role. Transitional metals may trigger oxidative stress and inflammatory responses through generation of reactive oxygen species (ROS) [15]. In toxicological studies, Wistar Kyoto rats had lower ANN and LnrMSSD following Nickel exposure and these effects were suppressed when inhibitors of inflammation were injected [16]. Additionally, peripheral injection of pro-inflammatory cytokines induced decreased HRV parameters among BALB/c mice [17]. In epidemiological studies, inflammation has been consistently associated with both metals exposure as well as alterations in cardiac autonomic functions. However, no study has examined their casual relationships, which may have important implications for knowing the underlying mechanisms and treatment for cardiovascular disease.

To date, several genetic variants associated with cardiac autonomic repose have been identified through genome-wide association studies (GWASs) [18-19]. Despite the success of GWAS in understanding the genetic components of cardiovascular effects, a large proportion of the causality is still unclear, such as gene-environment interactions. Recent studies have suggested that epigenetic mechanisms, especially DNA methylation may play an important role in regulation of gene expression in relation to cardiovascular responses. For example, the associations between PM2.5 exposure and DNA methylation of the iNOS gene were consistently observed [20-21]. A limitation of previous research is that these studies focused on gene-specific methylation analyses and other potential candidate genes across the whole genome were omitted. The epigenome-wide association study (EWAS), however, is a high-throughput approach to detect epigenetic variants across the whole genome, much like GWAS studies for genetic variations. The recently released Illumina 450K Infinium Methylation BeadChip provides a powerful approach to quantify DNA methylation levels at over 480,000 CpG across the whole genome and becomes more and more popular in EWAS [22].

Several studies have consistently reported higher risks of cardiac autonomic dysfunction among welders [23-24]. In addition, welders also have higher risks of exposure to various types of metals existed in the
welding fumes as compared with general population. Therefore, in the studies included within this thesis, we sought to identify the hazardous metal components, within the metals mixture, which were associated with AC or DC changes as well as to investigate the potential mechanism of these effects through inflammation effects and epigenetic variations among a population of welders. Our research differs from previous studies in several ways. First, we conducted model selection procedures for identifying significant metal components, which could allow us to account for the potential correlations between different metal spices. Second, we used two novel and more precise indexes – AC and DC as measurements of cardiac autonomic responses. Third, we examined causal relationships between metals exposure, inflammation and AC or DC changes. Finally, we conducted the first EWAS to examine epigenetic variations associated with AC or DC.

In chapter 2, we examined associations of 16 types of metals exposure with AC or DC values. We fitted L1-penalized linear mixed-effects models with Lasso to identify significant metal components associated with AC or DC changes. We then fitted co-pollutants models including only “significant” metals to explore the exposure-response relationships. In chapter 3, we went on to examine whether effects on AC or DC of metals exposure were mediated through inflammation using the linear mixed effects models. We conducted mediation analyses by fitting the mediation models, in which AC or DC values were used as outcome variables, urinary metals concentrations were used as exposure variables and serum inflammatory cytokines levels were checked as mediators. We estimate the direct and indirect effects in both single-pollutant and co-pollutant models. In chapter 4, we conducted the EWAS to identify genetic locations at which DNA methylation level changes were associated with AC or DC quantities. We fitted separate linear mixed models and we corrected for multiple testing using both false discovery rate (FDR) and the Bonferroni correction.

The goal of this thesis is to expand current knowledge on relationships between metals exposure and cardiac autonomic responses as well as the potential underlying biological mechanisms. We hope findings
from the current thesis will have implications on future research and regulations, especially in the occupational health.

REFERENCES


CHAPTER 2
Application of ℓ(1)-penalized linear mixed-effects model with Lasso to identify metal components associated with cardiac autonomic responses among welders: a repeated measures study.

Jinming Zhang¹
Jennifer M. Cavallari⁴
Shona C. Fang⁵
Marc G. Weisskopf³,²
Xihong Lin³
Murray A. Mittleman²,⁶
David C. Christiani¹,²,⁷
*Corresponding author

Affiliations:
1. Department of Environmental Health, Harvard T. H. Chan School of Public Health, Boston, USA
2. Department of Epidemiology, Harvard T. H. Chan School of Public Health, Boston, USA
3. Department of Biostatistics, Harvard T. H. Chan School of Public Health, Boston, USA
4. Department of Community Medicine and Health Care, University of Connecticut Health Center, Farmington, USA
5. Department of Epidemiology, New England Research Institute, Watertown, USA
6. Cardiovascular Epidemiology Research Unit, Beth Israel Deaconess Medical Center/Harvard Medical School, Boston, USA
7. Pulmonary and Critical Care Division, Massachusetts General Hospital/Harvard Medical School, Boston, USA

Keywords: Metal, Deceleration, Acceleration, Heart Rate, Welding
ABSTRACT

Background: Environmental and occupational exposure to metals is ubiquitous worldwide and understanding the hazardous metal components in this complex mixture is essential for environmental and occupational regulations.

Objective: To identify hazardous components from metal mixtures that are associated with alterations in cardiac autonomic responses.

Methods: Urinary concentrations of 16 types of metals were examined simultaneously and “acceleration capacity” (AC) and “deceleration capacity” (DC), indicators of cardiac autonomic effects, were quantified from electrocardiogram recordings among 54 welders. We fitted linear mixed-effects models with LASSO to identify metal components that are associated with AC and DC changes. The Bayesian Information Criterion (BIC) was used as the criterion for model selection procedures.

Results: Mercury and chromium were selected for the DC analysis, whereas mercury, chromium and manganese were selected for AC analysis through the LASSO approach. When we fitted the linear mixed-effects models with “selected” metal components only, the effect of mercury remained significant. Every 1μg/L increase in urinary mercury was associated with -0.58ms (-1.03, -0.13) changes in DC and 0.67ms (0.25, 1.10) changes in AC.

Conclusion: Our study suggests that exposure to several heavy metals are associated with imbalance between sympathetic and parasympathetic activities. Our findings should be replicated in future studies with larger sample sizes.
Introduction

In recent years, toxic metal pollution has become one of major environmental problems across the globe due to increase in industrialization and anthropogenic activities. There has been increasing concern over the impact of metals exposure on cardiovascular diseases (CVD)[1-3]. Studies have shown that alterations in cardiac autonomic responses might be one of the potential mechanisms of cardiovascular effects of metals exposure [4-5]. The imbalance between sympathetic and parasympathetic activities, which modulate heart rate, is associated with increased cardiovascular morbidity and mortality [6-7].

Conventional heart rate variability (HRV) parameters, including standard deviation of NN intervals (SDNN), the root mean square of heartbeat interval differences (RMSSDs), high frequency (HF) and low frequency (LF), have been widely used as indicators of cardiac autonomic responses. However, recent studies suggested that these HRV parameters might not be accurate as they failed to account for heart rate, a potential confounder [8]. One possibility might be the phase-rectified signal averaging (PRSA) approach introduced by Bauer et al. [9]. Using this method, the deceleration and acceleration were separately characterized through two novel markers: “acceleration capacity” (AC) and “deceleration capacity” (DC). DC provides a measure of parasympathetic modulation of heart rate. Bauer et al. reported that impaired DC was a strong predictor of cardiovascular mortality in a study among patients after myocardial infarction and it is more precise over conventional HRV estimators [9-10].

Welders are exposed to various types of metals from welding fumes. Welding materials often consist of mixtures of metals, such as iron, manganese, chromium and nickel [11]. Metal-rich welding fumes are small particles consisting of mostly vaporized metals that are suspended in air [12]. Previous studies have reported a higher risk of cardiac autonomic dysfunction associated with fine particulate matters (PM2.5) exposure from welding fumes [13-14]. However, few studies have examined the health effects of specific metal components. Identifying the hazardous metal species responsible for the adverse cardiac effects will have important implications for regulations in occupational settings. Therefore, in the current study, we examined the association between 16 metal species and AC and DC values. To address the challenge of
disentangling effects of correlated metals in a mixture such as welding fume, we conducted novel model selection procedures to identify metal components that are significantly associated with alterations in cardiac autonomic functions.

**METHODS**

**Study population**

The study population included a total of 54 welders from a local boilermaker union in Quincy, MA. Participants were recruited and monitored over four sampling occasions in the summer and winter months: June 2010, January 2011, June 2011 and June 2012, periods of boilermaker “off-season” at the union welding school and they were allowed to participant multiple times within the four sampling occasions. All participants were male workers who a) were 18 years of age or older; b) were union apprentice or journeyman; c) were free of cardiovascular disease at first entry; d) provided urine and electrocardiogram (ECG) samples at least once over the four sampling periods.

We monitored each participant for approximate six hours during a “work-shift” at each sampling occasion. The major tasks were welding, cutting and grinding activities at a union welding school. Each participant provided a urine sample and resting ECG recordings at both prior (baseline) and post work.

We collected questionnaires on demographics including age, height, weight, smoking status, medical history and medication use from participants at baseline. We obtained informed written consent from participants prior to participation. The Harvard T. H. Chan School of Public Health Institutional Review Board approved our study protocol.

**Electrocardiographic recording and sample analysis**

During each sampling occasion, participants were fitted with a 7-lead ambulatory ECG Holter monitor.

We collected 12-minutes resting ECG recordings from each participant at both prior and post work-shift. During the 12-minutes resting period, participants were asked to remain seated and quiet; walking,
talking or eating was not allowed, as AC and DC are sensitive to these activities. The ECGs samples were then sent to the Cardiovascular Epidemiology Research Unit of Beth Israel Deaconess Medical Center (Boston, Massachusetts, USA), where trained technicians blinded to exposure status processed and analyzed these samples. The methods of processing ECGs samples for AC and DC analysis have been discussed in our previous study [15]. For ECGs sample analysis, AC and DC quantities were computed and summarized every 5-minutes through the phase-rectified signal averaging (PRSA) method, as described by Bauer et al. In brief, to compute DC values, RR intervals longer than the immediate preceding interval were defined as anchors; to compute AC values, RR intervals shorter than the immediate preceding interval were defined as anchors. Segments of interval data that had the same size around these anchors were identified and aligned at those anchors. Upon alignment of all segments, RR intervals at all defined anchors (X0), immediate preceding (X1) and following the anchors (X-1) were averaged separately. The quantities of AC or DC were obtained by computing the difference between the sum of X0 and X1 and the sum of (X-1) and (X-2) [9]. To account for acclimation, the first two minutes of the 12-minutes resting ECG recordings were discarded and the remaining 10-minutes recordings were summarized as two non-overlapping 5-minute AC or DC values (3th-7th and 8th-12th minute intervals). We collected a total of 280 5-minute AC or DC values.

**Measurement of urinary metals and creatinine**

During each sampling occasion, participants provided urine samples at both baseline and post-exposure. A total of 171 samples were collected during the four sampling occasions. Each urine sample was collected with a 120 ml sterile urine collection cup and temporarily kept on ice. Then an approximate 12 ml aliquot of urine was transferred to 15 ml Falcon tubes for storage in a freezer at -20 °C. The urine samples were sent to Brooks Rand Labs where urinary concentrations of 16 types of metals were determined. The inductively coupled plasma mass spectrometry (ICP-MS) using a modified EPA 1638 method was used to analyze aluminum (Al), copper (Cu), manganese (Mn), lead (Pb), zinc (Zn), and
cadmium (Cd) concentrations, while a modified EPA 1638 method incorporated the Dynamic Reaction Mode technology was used for iron (Fe), vanadium (V), chromium (Cr) and nickel (Ni) analysis. For quality control purpose, duplicate, matrix spike and matrix spike duplicate analyses as well as NIST SRM 1643e (certified reference material with trace elements in natural water) were used. The method detection limit (MDL) for urinary metals measurements ranged from 0.01 to 3.30μg/L. In addition, urinary creatinine levels were also determined.

**Statistical analysis**

In this study, we examined urinary concentrations of 16 types of metals - Cr, Fe, Cu, Mn, Ni, Pb, V, Zn, Cd, As, Mg, Hg, Co, Mo, Se and Al, including metals that are commonly found in the welding fumes as well as trace metals that have been associated with cardiovascular diseases from previous research. A group of covariates were controlled for including age (a continuous variable in years), body mass index (BMI; calculated as weight in kilograms divided by height squared in meters), current smoking status (categorized into two groups: non-smoker, former smoker or current smoker), time (baseline or post-work) and season (summer or winter when each sampling occasion occurred). These variables have been reported as predictors of cardiac autonomic responses. A variable “time in day” was also adjusted for as AC and DC might exhibit a circadian pattern throughout the day [16]. In addition, urinary creatinine level was included in the model as a covariate as studies suggested the potential bias might be introduced when using the creatinine-standardized metal concentration (the ratio of metal concentrations over creatinine levels) [17]. All these variables were included in the models as non-penalized items. In other words, they were “fixed” variables and were not “selected” through variable selection procedures.

Tibshirani [18] introduced the idea of “least absolute shrinkage and selection operator” (lasso), which is to “minimize the residual sum of squares subject to the sum of the absolute value of the coefficients
being less than a constant”. Lasso retains satisfactory features of both subset selection and ridge regression. By applying $L_1$ penalty, lasso shrinks the estimates of regression coefficients and results in many regression coefficients being exactly equal to zero, whereas a few other coefficients being non-zero with little shrinkage due to its sparsity-inducing properties. We conducted linear mixed-effects models with lasso to further account for correlations of repeated measurements from the same subject. The linear mixed-effects models with random subject-specific intercepts assessing metals exposure on AC or DC changes ($Y_{ij}$) were fitted as follows:

$$Y_{ij} = X_i \beta + \gamma_1 \text{Age}_{ij} + \gamma_2 \text{BMI}_{ij} + \gamma_3 \text{Time in day}_{ij} + \gamma_4 \text{smoking status}_{ij} + \gamma_5 \text{Time}_{ij} + \gamma_6 \text{Season}_{ij} + b_i + \epsilon_{ij}$$

Where $\epsilon_{ij} \sim N(0, \sigma^2)$ and $b_i \sim N(0, \sigma_b^2)$, $\beta = (\beta_{i1}, \ldots, \beta_{ik})^T$ indicates a vector of regression coefficients of metals components vector $X_i = (X_{i1}, \ldots, X_{ik})$ that are “selected” via lasso with $L_1$-penalty.

When $L_1$ penalty is applied, a non-negative tuning parameter $\lambda$ is used to determine the amount of shrinkage or penalization. For example, a). when $\lambda$ equals to 0, there is minimum shrinkage and the model includes all metal components; b). when $\lambda$ is larger than certain value $K$, there is infinite shrinkage and the model includes no metal component, in other words, the regression coefficients of all metal components are zero; c). when $\lambda$ is between 0 and $K$, some of the regression coefficients shrunk to zero. Hence, variable selection is made possible by optimizing the value of $\lambda$. In this study, we used the Bayesian Information Criterion (BIC) [19] as the criterion for model selection and optimal value of $\lambda$ was achieved by minimizing BIC. In addition, we also conducted linear mixed-effects models to estimate pairwise correlations between metal components. All statistical analyses were performed with SAS v 9.3 (SAS Institute Inc, NC) and R 3.2.2 (R Core Team 2015).
Results

Demographic descriptions of the study population are summarized in Table 1.1. Among 54 male welders, there were 22(40.7%) current smokers and 32(59.3%) former smokers or non-smokers. The average age was 41.6 (ranged from 21.7 to 71.2 years). The average BMI was 28 kg/m². The median urinary metals concentrations ranged from 0.18ug/L to 77.55mg/L at baseline and 0.19ug/L to 82.30mg/L at post work. The distribution of urinary metals concentrations was highly skewed [Table 2.1].

Table 1.1: Demographics of study populations at first-entry (N=54).

<table>
<thead>
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<th>Characteristics</th>
<th>N(%) or Mean ± SD</th>
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<tr>
<td>Male</td>
<td>54(100)</td>
</tr>
<tr>
<td>Age(years)</td>
<td>41.6(12.4)</td>
</tr>
<tr>
<td></td>
<td>Range 21.7-71.2</td>
</tr>
<tr>
<td>BMI(kg/m²)</td>
<td>28 ±4</td>
</tr>
<tr>
<td>Race</td>
<td></td>
</tr>
<tr>
<td>Caucasian</td>
<td>50(92.6)</td>
</tr>
<tr>
<td>African American</td>
<td>2(3.7)</td>
</tr>
<tr>
<td>Hispanic</td>
<td>2(3.7)</td>
</tr>
<tr>
<td>Asian</td>
<td>0(0)</td>
</tr>
<tr>
<td>Current smoker</td>
<td>22(40.7)</td>
</tr>
<tr>
<td>Acceleration Capacity(ms)</td>
<td>-6.29(3.62)</td>
</tr>
<tr>
<td>Deceleration Capacity(ms)</td>
<td>7.62(3.23)</td>
</tr>
</tbody>
</table>

The relationship between λ and model BIC, the criterion for optimizing λ, is shown in Figure 1.1. For DC analysis, there was maximum shrinkage when λ=20; the model with the smallest BIC (λ=9) included mercury and chromium only and the regression coefficients of all the other metal components were zero. For AC analysis, the model with the smallest BIC (λ=6) included manganese in addition to mercury and chromium and there was maximum shrinkage when λ=21.
Table 2.1: Summaries of urinary metals concentrations at pre and post shift.

<table>
<thead>
<tr>
<th>Metals</th>
<th>Pre-shift</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th>Post-shift</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>P5</td>
<td>P25</td>
<td>P50</td>
<td>P75</td>
<td>P95</td>
<td>Mean</td>
<td>P5</td>
<td>P25</td>
<td>P50</td>
<td>P75</td>
</tr>
<tr>
<td>Chromium(ug/L)</td>
<td>0.30</td>
<td>0.10</td>
<td>0.12</td>
<td>0.22</td>
<td>0.41</td>
<td>0.67</td>
<td>0.45</td>
<td>0.10</td>
<td>0.16</td>
<td>0.27</td>
<td>0.46</td>
</tr>
<tr>
<td>Iron(mg/L)</td>
<td>0.23</td>
<td>0.03</td>
<td>0.09</td>
<td>0.18</td>
<td>0.36</td>
<td>0.55</td>
<td>0.22</td>
<td>0.03</td>
<td>0.10</td>
<td>0.20</td>
<td>0.30</td>
</tr>
<tr>
<td>Copper(ug/L)</td>
<td>10.51</td>
<td>2.63</td>
<td>5.55</td>
<td>9.06</td>
<td>14.20</td>
<td>22.00</td>
<td>11.36</td>
<td>2.73</td>
<td>6.17</td>
<td>9.53</td>
<td>14.10</td>
</tr>
<tr>
<td>Manganese(ug/L)</td>
<td>2.23</td>
<td>0.40</td>
<td>1.18</td>
<td>1.99</td>
<td>2.87</td>
<td>4.72</td>
<td>2.52</td>
<td>0.50</td>
<td>1.51</td>
<td>2.11</td>
<td>3.49</td>
</tr>
<tr>
<td>Nickel(ug/L)</td>
<td>1.63</td>
<td>0.50</td>
<td>0.66</td>
<td>1.23</td>
<td>2.26</td>
<td>3.86</td>
<td>1.79</td>
<td>0.50</td>
<td>0.95</td>
<td>1.52</td>
<td>2.39</td>
</tr>
<tr>
<td>Lead(ug/L)</td>
<td>0.51</td>
<td>0.08</td>
<td>0.23</td>
<td>0.36</td>
<td>0.77</td>
<td>1.27</td>
<td>0.70</td>
<td>0.10</td>
<td>0.31</td>
<td>0.49</td>
<td>0.89</td>
</tr>
<tr>
<td>Vanadium(ug/L)</td>
<td>0.26</td>
<td>0.10</td>
<td>0.10</td>
<td>0.18</td>
<td>0.40</td>
<td>0.69</td>
<td>0.24</td>
<td>0.10</td>
<td>0.10</td>
<td>0.22</td>
<td>0.36</td>
</tr>
<tr>
<td>Zinc(mg/L)</td>
<td>0.48</td>
<td>0.07</td>
<td>0.19</td>
<td>0.39</td>
<td>0.65</td>
<td>1.10</td>
<td>0.46</td>
<td>0.09</td>
<td>0.23</td>
<td>0.39</td>
<td>0.61</td>
</tr>
<tr>
<td>Cadmium(ug/L)</td>
<td>0.34</td>
<td>0.07</td>
<td>0.09</td>
<td>0.20</td>
<td>0.42</td>
<td>0.97</td>
<td>0.32</td>
<td>0.07</td>
<td>0.12</td>
<td>0.19</td>
<td>0.32</td>
</tr>
<tr>
<td>Arsenic(ug/L)</td>
<td>32.22</td>
<td>2.50</td>
<td>4.53</td>
<td>9.58</td>
<td>19.40</td>
<td>128.56</td>
<td>24.06</td>
<td>3.19</td>
<td>6.68</td>
<td>12.50</td>
<td>27.70</td>
</tr>
<tr>
<td>Magnesium(mg/L)</td>
<td>87.29</td>
<td>19.00</td>
<td>38.40</td>
<td>77.55</td>
<td>114.00</td>
<td>213.00</td>
<td>89.63</td>
<td>15.70</td>
<td>52.90</td>
<td>82.30</td>
<td>114.00</td>
</tr>
<tr>
<td>Mercury(ug/L)</td>
<td>0.71</td>
<td>0.10</td>
<td>0.21</td>
<td>0.52</td>
<td>1.01</td>
<td>2.21</td>
<td>0.69</td>
<td>0.07</td>
<td>0.21</td>
<td>0.41</td>
<td>0.74</td>
</tr>
<tr>
<td>Cobalt(ug/L)</td>
<td>0.49</td>
<td>0.35</td>
<td>0.40</td>
<td>0.40</td>
<td>0.52</td>
<td>0.78</td>
<td>0.52</td>
<td>0.32</td>
<td>0.40</td>
<td>0.44</td>
<td>0.57</td>
</tr>
<tr>
<td>Molybdenum(ug/L)</td>
<td>50.28</td>
<td>6.50</td>
<td>23.80</td>
<td>38.70</td>
<td>60.90</td>
<td>147.00</td>
<td>66.15</td>
<td>12.60</td>
<td>37.60</td>
<td>52.70</td>
<td>80.50</td>
</tr>
<tr>
<td>Selenium(ug/L)</td>
<td>63.74</td>
<td>12.20</td>
<td>35.90</td>
<td>56.30</td>
<td>83.50</td>
<td>159.00</td>
<td>75.49</td>
<td>21.40</td>
<td>44.70</td>
<td>66.71</td>
<td>96.90</td>
</tr>
</tbody>
</table>
When we fitted the linear mixed-effects models with fixed covariates and “selected” metal components only, there were negative associations of urinary mercury and chromium levels with DC, whereas there were positive associations of urinary mercury, chromium and manganese levels with AC. However, only the effects of mercury exposure exhibited statistical significance and effects of chromium and manganese exposure were marginally significant. A 1μg/L increase in urinary mercury was associated with -0.58ms (-1.03, -0.13) change in DC and 0.67ms (0.25, 1.10) change in AC [Table 3.1]. The correlation coefficients of urinary mercury and chromium, chromium and manganese as well as mercury and manganese that were estimated through linear mixed-effects models were 0.46, 0.37 and 0.29, respectively.

**Table 3.1: Effects of co-pollutants exposure on AC or DC changes.**

<table>
<thead>
<tr>
<th>Metal</th>
<th>DC</th>
<th>AC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hg(μg/L)</td>
<td>Coefficient: -0.576</td>
<td>Coefficient: 0.673</td>
</tr>
<tr>
<td></td>
<td>95% CI: -1.028, -0.125</td>
<td>95% CI: 0.249, 1.097</td>
</tr>
<tr>
<td></td>
<td>p-value: 0.013</td>
<td>p-value: 0.002</td>
</tr>
<tr>
<td>Cr(μg/L)</td>
<td>Coefficient: -0.479</td>
<td>Coefficient: 0.531</td>
</tr>
<tr>
<td></td>
<td>95% CI: -1.071, 0.111</td>
<td>95% CI: 0.025, 1.087</td>
</tr>
<tr>
<td></td>
<td>p-value: 0.100</td>
<td>p-value: 0.061</td>
</tr>
<tr>
<td>Mn(μg/L)</td>
<td>0.192</td>
<td>0.032</td>
</tr>
<tr>
<td></td>
<td>0.415</td>
<td>0.093</td>
</tr>
</tbody>
</table>
Discussion

Overall our findings suggested that exposure to airborne metals were associated with adverse cardiac autonomic responses. We used linear mixed-effects models with LASSO shrinkage method to identify metal components that might be associated with AC or DC. We observed that urinary mercury and chromium concentrations were negatively associated with DC, whereas urinary mercury, chromium and manganese concentrations were positively associated with AC. The significant effects of mercury exposure on AC and DC persisted in the co-pollutant models, while effects of chromium and manganese yielded marginal significance. Our findings suggested that metals exposure might induce imbalance between sympathetic and parasympathetic activities.

Mercury is classified as three major groups: elemental mercury, inorganic mercury compounds and organic mercury. To date, several biomarkers have been used to assess mercury exposure, such as hair, blood and urine samples [20-21]. Mercury levels in blood and hair are indicators of organic mercury exposures, whereas urinary mercury concentration mainly reflects exposure to elemental or inorganic forms of mercury. In the current study, we used urinary mercury concentrations to estimate medium-term mercury exposure, as it has a half-life of approximately 1-3 months in urine. The baseline median value (0.52μg/L) was similar to U.S. population medians from National Health [22] and Nutrition Examination Survey (0.48μg/L) and 17.5% lower than values (0.63μg/L) from a study in a dental professionals cohort [23].

While mercury has long been considered as an environmental and occupational neurotoxicant, there has been increasing concern about cardiotoxic effects of mercury exposure. Several studies have documented the role of organic mercury exposure in triggering cardiac autonomic responses. For example, Lim et al. [24] examined hair mercury levels and heart rate variability in a cross-sectional study among 1589 residents living near industrial complexes in South Korea, it was found that an 1 ppm increase in hair mercury concentration was associated with 8.4% decline in high frequency (HF) parameter. Yaginuma-Sakurai et al. [25] conducted a clinical trial where 27 participants in the intervention group were
instructed to consume bigeye tuna and swordfish once a week for 14 weeks, while 27 participants in the control group continued usual diets. Compared with controls, participants in the intervention group had significantly higher ratio of low frequency to high frequency (LF/HF) at the end of the study. On the other hand, epidemiological research investigating the cardiovascular effects of elemental or inorganic mercury exposure has mainly focused on blood pressure [26-27]. To our knowledge, this is the first study reporting the association of element or inorganic mercury exposure and cardiac autonomic responses among human population.

Furthermore, it is notable that these welders might be exposed to elemental or inorganic mercury from ambient environment rather than occupational settings, as mercury is not a common welding fume component. Studies have reported ubiquitous exposure to inorganic forms of mercury among population in US and Canada [28-29]. Elemental mercury is often used in products like thermometers and dental amalgam [30-31]. In the general population, elemental mercury from dental amalgam is one of the major sources of mercury exposure. Inorganic mercury compounds have been widely used in batteries or organic chemicals. In addition, urinary mercury levels could also change due to high fish consumption [32].

On the other hand, chromium and manganese are both common components exist in the welding fumes. These vaporized metal components become small particles that could suspend and react with oxygen in the air throughout the welding process. Chromium can be found in two forms: trivalent chromium [Cr(III)] or hexavalent chromium[Cr(VI)]. Cr(VI) is a well-established environmental pollutant and is much more toxic than Cr(III) [33]. Inhalation, digestion and dermal absorption were the main pathway of occupational chromium exposure. In the current study, we measured short-term Cr(VI) exposure through urinary Cr(VI) concentrations, which reflect exposure over approximately 1-2 days [34]. We observed a significant association between occupational chromium exposures and impaired cardiac autonomic effects. Similar to our findings, Cavallari et al. [35] found that airborne Cr(VI) exposure was associated with
reduced nocturnal rMSSD among this welder population. However, evidence from epidemiological studies was very limited and the cardiotoxicity of Cr(VI) exposure warrants further research.

In addition, urinary manganese concentrations were used to estimate recent airborne manganese from welding fumes among these welders [36]. Previous studies have documented the association of manganese exposure and annual cardiovascular mortality as well as manganese being a modifier of the association between PM2.5 exposure and hospital admissions for CVD [37]. Consistent with research on airborne manganese among manganese alloy workers [38] and boilermaker construction workers [35], our studies suggested that urinary manganese levels were associated with impaired cardiac autonomic responses.

Although the underlying mechanisms of metals exposure on cardiovascular effects are still under investigation, studies suggested that oxidative stress, inflammatory responses and enzymatic inhibition [39-41] might play a role. Transition metals, such as mercury, chromium and manganese, may undergo redox cycling reactions and induce oxidative stress through enhanced generation of reactive oxygen species (ROS) [39]. Low concentrations of mercury exposure may induce decreased NO bioavailability due to oxidative stress and further promote endothelial dysfunction in both resistance and conductance arteries in male Wistar rats [42]. Furthermore, decreased HRV parameters (SDNN, SD1 and SD2) following peripheral injection of Interleukin 6, a pro-inflammatory cytokine, were documented in another toxicological study [41]. Taken together, future studies examining oxidative stress and inflammation as the mediators linking metals exposure and cardiovascular effects may be essential for illustrating the underlying causal pathway.

To our knowledge, this is the first repeated measurements study investigating metal components exposure and cardiac autonomic effects. We used linear mixed-effects models with LASSO for model selection procedures and this approach has several advantages over conventional methods. Typically, researchers examined the effects of metal components by fitting a model including all components simultaneously. However, collinearity issues due to high correlations among metal components may induce inflated
variances of the estimated regression coefficients and sap the statistical power [43]. Another commonly used approach-stepwise regression may have similar problems in the presence of collinearity as well as yield biased coefficients that need shrinkage [18].

When we fitted “selected” metals only in the co-pollutant model, the effects of chromium and manganese were only marginally significant. This may due to the relatively small sample size and the modest correlations among these metal components. It is also notable that metals may often induce antagonistic, additive or synergistic responses in the presence of other metal components. For example, Papp et al. observed synergistic neurotoxicity of combined exposure mercury, manganese and lead among male Wistar rats [44]. While the exact relationships between mercury, chromium and manganese in triggering cardiac autonomic effects were unclear, it is possible that model misspecification may also occur in this study as we assumed independent relationships among these components in the co-pollutant model.

Several other limitations to our study should be considered. First, urinary concentrations of some metals [45-46] may not be ideal for assessing metals exposures and non-differential exposure misclassification may be possible. Second, measurement errors may also occur due to individual variability in excretion of metal and creatinine in urine. Third, there may be healthy worker effect (HWE) if these welders tend to be healthier compared with non-working population. Finally, our study had a relatively small sample size and results from our study need to be replicated in additional cohorts of metal-exposed workers.

ACKNOWLEDGEMENTS

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CHAPTER 3
Airborne metal exposure, cardiac autonomic responses and inflammation among welders-A mediation analysis.

Jinming Zhang\textsuperscript{1}
Marie-Abèle Bind\textsuperscript{8}
Jennifer M. Cavallari\textsuperscript{4}
Shona C. Fang\textsuperscript{5}
Marc G. Weisskopf\textsuperscript{1,2}
Xihong Lin\textsuperscript{3}
Murray A. Mittleman\textsuperscript{2,6}
David C. Christiani\textsuperscript{1,2,7}
*Corresponding author

Affiliations:

1. Department of Environmental Health, Harvard T. H. Chan School of Public Health, Boston, USA
2. Department of Epidemiology, Harvard T. H. Chan School of Public Health, Boston, USA
3. Department of Biostatistics, Harvard T. H. Chan School of Public Health, Boston, USA
4. Department of Community Medicine and Health Care, University of Connecticut Health Center, Farmington, USA
5. Department of Epidemiology, New England Research Institute, Watertown, USA
6. Cardiovascular Epidemiology Research Unit, Beth Israel Deaconess Medical Center/Harvard Medical School, Boston, USA
7. Pulmonary and Critical Care Division, Massachusetts General Hospital/Harvard Medical School, Boston, USA
8. Department of Statistics, Faculty of Arts and Sciences, Harvard University, Cambridge, USA

Keywords: Metal, Resting deceleration capacity, Resting acceleration capacity, Heart rate, Inflammation, Mediation analysis
Abstract

Background: Metals exposure has been associated with cardiac autonomic dysfunction. While the underlying mechanisms are still being explored, studies have suggested that inflammatory responses may play a role.

Objective: We aimed to investigate whether inflammatory responses mediate the effects of metals exposure on cardiac autonomic changes.

Methods: Sixty-four welders were recruited from a local boilermaker union during four sampling occasions between June 2010 and June 2012. We collected urine, blood and electrocardiogram (ECG) samples from participants at both prior and post-work. Urinary concentrations of 10 types of metals were determined. Serum inflammatory markers including C-reactive protein (CRP), interleukin 2 (IL-2), interleukin 6 (IL-6) and interleukin 8 (IL-8) were also examined. In addition, resting acceleration capacity (AC) and deceleration capacity (DC) values were quantified as measurements cardiac autonomic responses. We fitted longitudinal mediation models with random intercepts to examine both the direct and indirect effects of metals exposure on AC and DC mediated by inflammatory markers. Both single pollutant models and co-pollutant models were examined through the mediation analysis.

Results: There were significant negative direct effects of chromium and iron exposure on DC as well as significant positive direct effects on AC after adjusting for inflammatory markers. A 1μg/L increase in urinary chromium was associated with -1.84ms (-2.93, -0.76) change in DC and 1.76ms (0.59, 2.94) change in AC, whereas a 1mg/L increase in urinary iron was associated with -2.81ms (-4.98, -0.63) change in DC and 2.60ms (0.26, 4.94) change in AC. None of the indirect effects were statistically significant. The co-pollutant mediation models yielded similar results.

Conclusion: Airborne metal exposure may affect both acceleration and deceleration of heart rate. Our study does not support the hypothesis that inflammation might be on the pathway linking metals exposure and cardiac autonomic responses, but rather a direct effect.
INTRODUCTION

The association between airborne metals exposure and impaired cardiac autonomic functions has been well documented [1-3]. Cardiac autonomic dysfunction may occur in many diseases, such as diabetes[4], chronic obstructive pulmonary disease (COPD)[5] and hypertension[6]. Some conventional heart rate variability (HRV) parameters, such as standard deviation of NN intervals (SDNN), the root mean square of heartbeat interval differences (RMSSDs), high frequency (HF) and low frequency (LF), have been consistently used as indices of cardiac autonomic responses. However, recent studies have suggested that those parameters might not be precise, as they do not account for heart rate [7-8]. In addition, parameters like SDNN are measurements of both sympathetic and parasympathetic activities [9], making it difficult to elucidate biological mechanisms when used in research studies.

In 2006, Bauer et al. introduced the phase-rectified signal averaging (PRSA) method to quantify “acceleration capacity” (AC) and “deceleration capacity” (DC) [8]. By separately characterizing deceleration and acceleration of heart rate, AC and DC could successfully distinguish between sympathetic and parasympathetic factors that affect heart rate variability. Impaired DC has been demonstrated as a more powerful and precise predictor of cardiovascular mortality among patients after myocardial infarction[8,10] as compared with conventional HRV parameters as well as left ventricular ejection fraction, the “gold standard” in the clinical practice.

Welders are exposed to a wide variety of metals present in welding fumes throughout the welding process. Metal-rich welding fumes are formed when vaporized metals become small particles and are suspended in air [11]. The vaporized metal subsequently reacts with oxygen in the air and becomes oxidized. Inhalation of oxides of metals is the main route that these metals enter the human body. While the exact metal constituents of welding fumes may vary depending on the electrodes and welding types, the most common metal components in the welding fumes include chromium, nickel, iron, manganese, fluorides, zinc, copper, aluminum and cadmium [12]. Previous studies also reported an increased risk of cardiac autonomic dysfunction among welders [13-14].
Although the underlying biological mechanisms of cardiac autonomic changes from metals exposure are unclear, studies suggested that inflammatory responses may play a role [15-17]. In the current study, we examined 10 types of metals exposure measured using urinary metals concentrations as well as quantified AC and DC values among a sample of welders. Serum IL-2, IL-6 IL-8 and CRP levels were also determined, as their associations with cardiac autonomic changes have been reported in previous studies. We aimed to investigate the mediation effect of metals exposure and cardiac autonomic responses through the inflammatory responses.

METHODS

Study population

The Harvard Boilermakers Study is a prospectively enrolled open cohort study conducted among a population of welders from a local boilermaker union in Quincy, MA. Those welders primarily assemble or weld boilers in large power plants. In the current study, participants included 64 welders recruited from four sampling occasions in June 2010, January 2011, June 2011 and June 2012. Those welders were allowed participant in multiple sampling occasions. The inclusion criteria were: a) male welders≥ 18 years of age; b) unionized welders including both apprentice and journeyman; c) contributed urine, blood and ECG samples at least once during the four sampling occasions. The exclusion criteria were clinical diagnosed cardiovascular disease as determined by self-report prior to participation.

During each sampling occasion, participants were monitored at a union welding school on an approximate 6-hour workday. Their major occupational activities included welding, grinding and cutting tasks. We collected urine, blood and resting ECG recordings at both prior- (baseline) and post- work. We also collected self-administrated questionnaires including age, height, weight, current smoking status and/or smoking history, medical history and medication use during the past six months from each participant at baseline, and this information was updated at each sampling occasion. The Harvard T. H. Chan School of
Public Health Institutional Review Board reviewed and approved the study protocol and we obtained a written informed consent from each participant at each sampling occasion.

**Electrocardiographic recording and sample analysis**

During each sampling occasion, participants were fitted with a 7-lead ambulatory electrocardiogram (ECG) Holter monitor. We collected 12-minutes resting ECG recordings from each participant at both prior and post work. During the 12-minutes resting period, participants were asked to remain seated and quiet; walking, talking or eating was not allowed, as AC and DC are sensitive to these activities. The ECGs samples were then sent to the Cardiovascular Epidemiology Research Unit of Beth Israel Deaconess Medical Center (Boston, Massachusetts, USA), where trained technicians blinded to exposure status processed and analyzed these samples. The methods of processing ECGs samples for AC and DC analysis have been discussed in our previous study [18]. For ECGs sample analysis, AC and DC quantities were computed and summarized every 5-minute through the PRSA method, as described by Bauer et al. [8]. In brief, to compute DC values, RR intervals longer than the immediate preceding interval were defined as anchors; to compute AC values, RR intervals shorter than the immediate preceding interval were defined as anchors. Segments of interval data that had the same size around these anchors were identified and aligned at those anchors. Upon alignment of all segments, RR intervals at all defined anchors (X0), immediate preceding (X1) and following the anchors (X-1) were averaged separately. The quantities of AC or DC were obtained by computing the difference between the sum of X0 and X1 and the sum of (X-1) and (X-2) [8]. To account for acclimation, the first two minutes of the 12-minutes resting ECG recordings were discarded and the remaining 10-minutes recordings were summarized as two non-overlapping 5-minute AC or DC values (3th-7th and 8th-12th minute intervals). We collected a total of 290 5-minute AC or DC values.

**Measurement of urinary metals and creatinine**
During each sampling occasion, participants provided urine samples at both baseline and post-exposure. A total of 229 urine samples were obtained during the four sampling occasions. Each urine sample was collected with a 120 ml sterile urine collection cup and temporarily kept on ice. Then an approximate 12 ml aliquot of urine was transferred to 15 ml Falcon tubes for storage in a freezer at -20 °C. The urine samples were sent to Brooks Rand Labs (Brooks Applied Labs, WA) where urinary concentrations of ten types of metals were determined. The inductively coupled plasma mass spectrometry (ICP-MS) using a modified EPA 1638 method was used to analyze Al, Cu, Mn, Pb, Zn, and Cd concentrations, while a modified EPA 1638 method incorporated the Dynamic Reaction Mode technology was used for Fe, V, Cr and Ni analysis. For quality control purpose, duplicate, matrix spike and matrix spike duplicate analyses as well as NIST SRM 1643e (certified reference material with trace elements in natural water) were used. The method detection limit (MDL) for urinary metals measurements ranged from 0.01 to 3.30 μg/L.

As the creatinine-standardized metal concentration (the ratio between metal concentrations and creatinine levels) may introduce potential bias when used as an independent variable in the regression models [19-20], we adjusted all models for creatinine levels as a covariate in our study.

**Determination of inflammatory markers**

A total of 211 blood samples were collected from participants at baseline and post-exposure through venipuncture by a phlebotomist. Each blood collection tube was centrifuged for 10 minutes and a 1.5ml aliquot of plasma was transferred to a cryogenic tube for storage in a freezer maintained at -80 °C. For inflammatory markers analysis, the serum CRP levels were determined using multiplex electrochemiluminescence immunoassay through MULTI-SPOT® 96-well Human Vascular Injury Panel II assay (Meso Scale Discovery, Rockville MD), whereas the serum IL-2, IL-6 and IL-8 levels were examined through the MULTI-ARRAY® 96-well custom cytokine/chemokine assay. For quality controls (QC), duplicate samples were analyzed and samples were reanalyzed when the coefficient of variation (CV) was greater than 20%. In addition, we pooled the plasma to form a QC sample within each bath of samples and the blinded pooled QC samples accounted for ten percent [21] of each bath of samples. The
intra- and inter-assay CVs based on values of QC samples were calculated and they were all less than 20%.

**Statistical analysis**

In the first stage, we used separate linear mixed effects models with random intercepts to investigate the associations between individual metal exposure and 1) AC or DC values as well as 2) levels of inflammatory markers. The mixed effects models accounts for correlated measurements among welders who participated on multiple sampling occasions. As AC and DC might exhibit circadian variations, we controlled for a variable- time in day, indicating the time when the ECGs sample was collected. We also adjusted all models for cigarette smoking by including a variable-smoking status, which was categorized into three groups: current smoker, former smokers and non-smoker. Additional a priori selected covariates adjusted for in models were age, BMI and time (baseline or post-exposure). Linear mixed effects models were also used for estimating the pairwise correlations between metal species.

We also fitted longitudinal mediation models with random intercepts [22] to examine the effect of individual metal exposure (E$_{ij}$) on AC or DC (Y$_{ij}$) through changes in levels of inflammatory makers (M$_{ij}$). As when there is no association between exposure and mediator, the mediation pathway will not exist, our mediation analysis only included “significant” metals from the first stage. The following two linear mixed effects models were fitted simultaneously:

1) $M_{ij} = \alpha_0 + c_i + \alpha_1 E_{ij} + \alpha_2 Age_{ij} + \alpha_3 BMI_{ij} + \alpha_4 \text{Time in day}_{ij} + \alpha_5 \text{smoking status}_{ij} + \alpha_6 \text{Time}_{ij} + e_{ij}$

   where $e_{ij} \sim N(0, \sigma_m^2)$ and $c_i \sim N(0, \sigma_c^2)$

2) $Y_{ij} = \gamma_0 + b_i + \gamma_1 E_{ij} + \gamma_2 M_{ij} + \gamma_3 Age_{ij} + \gamma_4 BMI_{ij} + \gamma_5 \text{Time in day}_{ij} + \gamma_6 \text{smoking status}_{ij} + \gamma_7 \text{Time}_{ij} + e_{ij}$

   where $e_{ij} \sim N(0, \sigma_y^2)$ and $b_i \sim N(0, \sigma_b^2)$

The indirect effect (or the mediation effect) of metals exposure on cardiac autonomic function via inflammation was calculated through the product of $\alpha_1 \times \gamma_2$ and we used the delta method to estimate the
variance of the indirect effect: \( \text{Var}(\gamma_2)\alpha_1^2 + 2 \text{Cov}(\alpha_1, \gamma_2) \alpha_1 \gamma_2 + \text{Var}(\alpha_1) \gamma_2^2 \) \[22\]. The direct effect was estimated by \( \gamma_1 \). In addition, we conducted a co-pollutant mediation analysis by including all “significant” metals in the mediation models to examine mediation effect of single metal after adjusting for the other types of metals. We considered statistical significance as two-tailed \( p < 0.05 \). All statistical analyses were performed with SAS v 9.3 (SAS Institute Inc, NC).

**RESULTS**

The study population included 64 male welders, 27 (i.e. 42.2%) of them were “current smokers” and 54 (i.e. 84.4%) of them were white. Their mean age was 41.3 (SD=12.5) years and mean BMI was 28 (SD=5) kg/m\(^2\). The average DC was 7.37 ms (SD=3.61), whereas the average AC was -6.01 ms (SD=3.47). The mean concentrations (SD) of CRP, IL-2, IL-6 and IL-8 were 4.08 (5.07) mg/L, 2.81 (3.37) pg/mL, 27.05 (18.39) pg/mL and 18.83 (19.88) pg/mL respectively (Table 1.2). The CRP, IL-2, IL-6 and IL-8 concentrations were log-transformed to reduce the skewness in the statistical models.

**Table 1.2: Demographics of study populations (N=64).**

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>N(%) or Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>64(100)</td>
</tr>
<tr>
<td>Age(years)</td>
<td>41.3(12.5)</td>
</tr>
<tr>
<td>Range</td>
<td>21.2-71.2</td>
</tr>
<tr>
<td>BMI(kg/m(^2))</td>
<td>28 ±5</td>
</tr>
<tr>
<td>Race</td>
<td></td>
</tr>
<tr>
<td>White</td>
<td>54(84.4)</td>
</tr>
<tr>
<td>Black</td>
<td>4(6.3)</td>
</tr>
<tr>
<td>Hispanic</td>
<td>4(6.3)</td>
</tr>
<tr>
<td>Asian</td>
<td>2(3.1)</td>
</tr>
<tr>
<td>Current smoker</td>
<td>27(42.2)</td>
</tr>
<tr>
<td>Acceleration Capacity(ms)</td>
<td>-6.01*(3.57)</td>
</tr>
<tr>
<td>Deceleration Capacity(ms)</td>
<td>7.37(3.61)</td>
</tr>
<tr>
<td>CRP(mg/L)</td>
<td>4.08(5.07)</td>
</tr>
<tr>
<td>IL-2(pg/mL)</td>
<td>2.81(3.37)</td>
</tr>
<tr>
<td>IL-6(pg/mL)</td>
<td>27.05(18.39)</td>
</tr>
<tr>
<td>IL-8(pg/mL)</td>
<td>18.83(19.88)</td>
</tr>
</tbody>
</table>
The distribution of urinary metals concentrations was summarized in table 2.2. The median concentrations of urinary chromium and iron were 0.19μg/L and 0.13mg/L respectively at baseline and 0.25μg/L and 0.15 mg/L respectively at post-exposure.

We conducted linear mixed effects models to examine the relationship between individual metal exposure and AC and DC as well as inflammatory markers. There were significantly positive associations between urinary chromium or iron concentrations and AC as well as serum IL-6 levels. In addition, urinary chromium and iron concentrations were negatively associated with DC (Table 3.2).

Based on the results from linear mixed effects models, we examined the effects of chromium and iron exposures on AC or DC with IL-6 as a mediator. In the single pollutant models, we observed significantly negative direct effects of chromium or iron exposure on DC (Table 4.2) and significantly positive direct effects on AC (Table 5.2). A 1μg/L increase in urinary chromium was associated with -1.84ms (-2.93, -0.76) change in DC and 1.76ms(0.59,2.94) change in AC, whereas a 1mg/L increase in urinary iron was associated with -2.81ms(-4.98, -0.63) change in DC and 2.60ms (0.26,4.94) change in AC. The indirect effects of exposure to chromium or iron were -0.13(-0.30, 0.06) and -0.23(-0.56, 0.11) on DC as well as 0.18(-0.03,0.39) and 0.36(-0.06,0.77) on AC respectively. However, none of these indirect effects were statistically significant. We further checked the interaction terms of individual metal exposure with IL-6 levels, individual metal exposure with age, BMI, or smoking status as well as the interactions between these two types of metals, and none of them exhibited statistical significance (data not shown).

Furthermore, in the co-pollutant models, we observed similar results, although the absolute values of direct effects became smaller towards the null. Another difference was that the direct effect of iron exposure on AC was no longer significant when the model was adjusted for chromium. All indirect effects remained insignificant. The correlation between urinary chromium and iron concentrations estimated through linear mixed effects models was 0.46.
Table 2.2: Summaries of urinary metals concentrations at pre and post shift.

<table>
<thead>
<tr>
<th>Metals</th>
<th>Pre-shift</th>
<th></th>
<th>Post-shift</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>P5</td>
<td>P25</td>
<td>P50</td>
</tr>
<tr>
<td>Chromium(ug/L)</td>
<td>0.303</td>
<td>0.100</td>
<td>0.100</td>
<td>0.190</td>
</tr>
<tr>
<td>Iron(mg/L)</td>
<td>0.178</td>
<td>0.020</td>
<td>0.061</td>
<td>0.130</td>
</tr>
<tr>
<td>Copper(ug/L)</td>
<td>10.130</td>
<td>1.930</td>
<td>5.540</td>
<td>8.360</td>
</tr>
<tr>
<td>Manganese(ug/L)</td>
<td>2.284</td>
<td>0.450</td>
<td>1.230</td>
<td>2.240</td>
</tr>
<tr>
<td>Nickel(ug/L)</td>
<td>1.424</td>
<td>0.500</td>
<td>0.560</td>
<td>1.040</td>
</tr>
<tr>
<td>Lead(ug/L)</td>
<td>0.512</td>
<td>0.070</td>
<td>0.200</td>
<td>0.360</td>
</tr>
<tr>
<td>Vanadium(ug/L)</td>
<td>0.233</td>
<td>0.100</td>
<td>0.100</td>
<td>0.150</td>
</tr>
<tr>
<td>Zinc(mb/L)</td>
<td>0.439</td>
<td>0.059</td>
<td>0.221</td>
<td>0.373</td>
</tr>
<tr>
<td>Cadmium(ug/L)</td>
<td>0.333</td>
<td>0.070</td>
<td>0.080</td>
<td>0.160</td>
</tr>
</tbody>
</table>

Table 3.2: Association between individual metal exposure and changes in AC and DC and inflammatory markers

<table>
<thead>
<tr>
<th>Metal</th>
<th>AC</th>
<th>DC</th>
<th>CRP</th>
<th>IL-2</th>
<th>IL-6</th>
<th>IL-8</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>β (SE) P</td>
<td>β (SE) P</td>
<td>β (SE) P</td>
<td>β (SE) P</td>
<td>β (SE) P</td>
<td>β (SE) P</td>
</tr>
<tr>
<td>Cr(μg/L)</td>
<td>1.85(0.60) P0.002*</td>
<td>-1.96(0.54) &lt;0.001*</td>
<td>0.01(0.15)</td>
<td>0.946</td>
<td>0.29(0.17)</td>
<td>0.064</td>
</tr>
<tr>
<td>Fe(mg/L)</td>
<td>2.93(1.15) P0.012*</td>
<td>-2.89(1.06) 0.007*</td>
<td>-0.03(0.30)</td>
<td>0.913</td>
<td>0.27(0.26)</td>
<td>0.293</td>
</tr>
<tr>
<td>Mn(μg/L)</td>
<td>0.01(0.14)</td>
<td>0.944</td>
<td>-0.03(0.13)</td>
<td>0.847</td>
<td>0.03(0.03)</td>
<td>0.377</td>
</tr>
<tr>
<td>Cu(μg/L)</td>
<td>0.02(0.02)</td>
<td>0.303</td>
<td>-0.04(0.03)</td>
<td>0.055</td>
<td>0.01(0.01)</td>
<td>0.441</td>
</tr>
<tr>
<td>Ni(μg/L)</td>
<td>0.17(0.22)</td>
<td>0.442</td>
<td>-0.05(0.20)</td>
<td>0.797</td>
<td>0.02(0.06)</td>
<td>0.707</td>
</tr>
<tr>
<td>Pb(μg/L)</td>
<td>0.48(0.38)</td>
<td>0.206</td>
<td>-0.42(0.29)</td>
<td>0.152</td>
<td>0.08(0.09)</td>
<td>0.401</td>
</tr>
<tr>
<td>V(μg/L)</td>
<td>0.65(1.02)</td>
<td>0.522</td>
<td>-0.88(0.93)</td>
<td>0.334</td>
<td>0.13(0.25)</td>
<td>0.600</td>
</tr>
<tr>
<td>Zn(mg/L)</td>
<td>0.84(0.65)</td>
<td>0.195</td>
<td>-0.56(0.30)</td>
<td>0.068</td>
<td>0.26(0.17)</td>
<td>0.126</td>
</tr>
<tr>
<td>Cd(μg/L)</td>
<td>0.10(0.77)</td>
<td>0.901</td>
<td>-1.24(0.75)</td>
<td>0.101</td>
<td>0.39(0.25)</td>
<td>0.117</td>
</tr>
<tr>
<td>Al(μg/L)</td>
<td>0.05(0.04)</td>
<td>0.246</td>
<td>-0.02(0.04)</td>
<td>0.597</td>
<td>0.01(0.01)</td>
<td>0.533</td>
</tr>
</tbody>
</table>

† Separate linear mixed models were fitted and we adjusted all models for age, BMI, time, time_in_day, smoking status and urinary creatinine levels.
Table 4.2: Mediation effects of Cr or Fe exposure on AC or DC changes mediated by serum IL-6 in single-pollutant model.

<table>
<thead>
<tr>
<th>Metal</th>
<th>DC Direct effect(95% CI)</th>
<th>DC Indirect effect(95% CI)</th>
<th>AC Direct effect(95% CI)</th>
<th>AC Indirect effect(95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cr(μg/L)</td>
<td>-1.842(-2.925,-0.758)</td>
<td>-0.125(-0.304,0.055)</td>
<td>1.763(0.589,2.937)</td>
<td>0.179(-0.032,0.391)</td>
</tr>
<tr>
<td>Fe(mg/L)</td>
<td>-2.810(-4.980,-0.630)</td>
<td>-0.229(-0.569,0.109)</td>
<td>2.596(0.256,4.936)</td>
<td>0.356(-0.059,0.771)</td>
</tr>
</tbody>
</table>

Table 5.2: Mediation effects of Cr or Fe exposure on AC or DC changes mediated by serum IL-6 in co-pollutant model.

<table>
<thead>
<tr>
<th>Metal</th>
<th>DC Direct effect(95% CI)</th>
<th>DC Indirect effect(95% CI)</th>
<th>AC Direct effect(95% CI)</th>
<th>AC Indirect effect(95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cr(μg/L)</td>
<td>-1.645(-2.737,-0.554)</td>
<td>-0.084(-0.235,0.067)</td>
<td>1.573(0.383,2.764)</td>
<td>0.137(-0.049,0.322)</td>
</tr>
<tr>
<td>Fe(mg/L)</td>
<td>-2.210(-4.390,-0.040)</td>
<td>-0.154(-0.437,0.128)</td>
<td>2.003(-0.350,4.356)</td>
<td>0.261(-0.096,0.618)</td>
</tr>
</tbody>
</table>
DISCUSSION

In the current study, we used mediation analysis to examine the effects of metal exposures on changes in cardiac autonomic responses, as measured by AC and DC, and whether these effects were mediated through an inflammatory pathway. We observed significant direct effects of exposure to chromium and iron on AC and DC. However, the mediation effects were not statistically significant and our results did not support the hypothesis that systemic inflammation mediate these effects.

Urinary chromium concentration reflects short-term exposures, as the half-life of chromium in urine is usually 1-2 days [23]. While it has been suggested that dietary intake of chromium picolinate in a low dose might reduce the risk of diabetes and cardiovascular disease [24], environmental or occupational exposure to chromium may trigger adverse health effects, such as lung cancer, cardiovascular disease and renal injury [25]. The associations between chromium exposure and impaired cardiac autonomic as well as inflammatory responses were consistently reported in previous epidemiological studies. A study conducted among this welder population [26] observed a negative linear association between airborne chromium exposure and nocturnal rMSSD. In another occupational study, Riediker et al. [27] found that exposure to airborne Cr was associated with increased white blood cell count and IL-6 levels 15 hours following the Cr exposure among nonsmoking highway patrol officers.

The role of iron in triggering cardiovascular effects, however, is controversial. Sullivan[28] argued that the risk of CVD in men and postmenopausal women was much higher than that in premenopausal women due to premenopausal women having more depletion of iron stores as a result of menstrual bleeding. Muñoz-Bravo et al.[29] analyzed 55 studies investigating the relationship between iron levels and cardiovascular disease, they observed a fair amount of debate with 27 supporting the iron hypothesis, 20 reporting no evidence and 8 were against this hypothesis.

Results from our study, however, are consistent with some more recent studies, indicating that exposure to iron may be associated with adverse cardiac autonomic effects. In a cross-sectional study, Feng et al.
[30] examined 23 urinary metals concentrations and conventional HRV parameters among 2004 adult residents in Wuhan, China. They found that every 10-fold increase in urinary iron levels was associated with 12.22% lower HF and 8.25% lower SDNN. Using airborne iron levels to measure iron exposure, Wu et al. found that every 481.4 ng/m$^3$ increase in iron was associated with 8.11 ms declines in SDNN among taxi drivers in Beijing, China [31]. Hampel et al. conducted a meta-analysis to examine the relationships between long-term elemental PM metal components and high-sensitivity C-reactive protein (hsCRP) levels among 5 cohorts in Northern and Central Europe, they observed elevated hsCRP levels associated with both long term PM$_{10}$ iron and PM$_{2.5}$ iron [32].

In addition, the association between inflammatory responses and alterations in cardiac autonomic effects was also well documented. Hamaad et al. [33] found modest and negative associations between HRV parameters (SDNN and LF) and interleukin-6 (IL-6), CRP and white cell counts among 100 patients with acute coronary syndrome. Sloan et al. [34] also observed these associations in the same direction in a study among 757 healthy adults aged from 35 to 55. In this study, we hypothesized that airborne metal exposure may act as a trigger of impairment of cardiac autonomic effects through inflammation responses.

However, results from the current study did not provide evidence that metals exposure induce impaired cardiac autonomic effects through inflammatory responses. This might be due to the weak associations between changes in inflammatory markers and AC or DC. Among the inflammatory factors examined, IL-2, IL-6 and IL-8 belong to pro-inflammatory cytokines that are released by macrophages or T lymphocytes to promote systematic inflammation responses, while CRP is an acute phase reactant synthesized by liver following the secretion of IL-6[35]. It is notable that the ECGs samples were collected shortly (usually less than 15 minutes) after the collection of blood samples. Evidence from one study indicated that male BALB/c mice did not have significantly lower HRV parameters until 60 minutes following IL-6 injection compared with the controls [17]. Although there is insufficient evidence from human study suggesting the relevant time course, it is likely that we fail to capture the appropriate time window for the synthesized inflammatory factors to induce cardiovascular effects.
In fact, there has been debate over the causal-relationship between HRV and inflammatory responses. Tracey et al. [36] introduced the “anti-inflammatory reflex” hypothesis which described the role of autonomic nervous systems in regulation of host defense. Activation of vagal efferent fibers by the brain would further modulate inflammation to pathogens [36]. The brain may modulate the synthesis inflammatory cytokines, such as IL-6, through activating the vagal activities [36]. However, there was limited evidence from epidemiological studies to support this hypothesis. In fact, more studies suggested the importance of circulating inflammatory cytokines on influencing the cardiovascular regulatory system by activation of the corresponding cytokines receptors, especially in toxicological studies. For example, Chuang et al.[16] found that 72 hours following nickel exposure, there was significantly lower LnrMSSD and ANN among Wistar Kyoto rats and after those rats were given NAC and celecoxib, agents that could suppress oxidative stress and inflammatory responses, the effects of nickel exposure on HRV was significantly suppressed. Similar results were observed from another animal study by Hajiasgharzadeh et al. [17], there were significantly lower HRV parameters (SDNN, SD1 and SD2) following peripheral injection of 200 ng IL-6. In this study, we fitted the mediation models using inflammatory markers as mediators and AC or DC values as the response variables. The possibility of reverse causation, nevertheless, cannot be excluded in our study.

Furthermore, exposure to metals mixtures may induce antagonistic, additive or synergistic responses. For example, Moriwaki et al. [37] reported additive effects of iron and chromium as well as a synergistic interaction between chromium and copper on the formation of 8-hydroxy-deoxyguanosine (8-OHdG), a biomarker used for oxidative DNA damage measurement. Ashok et al. [38] observed synergistic effects of arsenic, cadmium and lead mixture on Alzheimer's disease (AD) like pathology and cognitive impairments in young rats. Taken together, it is possible that chromium and iron may act with other metal components and trigger adverse health effects through metals mixtures in a more complicated way.

To our knowledge, this is the first study to investigate the potential pathway of cardiovascular effects of metals exposure using the mediation analysis. Typically, researchers only reported binary associations of
metals exposure, inflammation and heart rate variability in cross-sectional studies. However, our study sought to illustrate the causal relationships between these associated factors and link them through mediation pathway. Additionally, urinary metals concentrations were used to estimate recent metals exposure, these estimates may provide more accurate measurements of the “internal” dose of exposure compares with the estimations from the history of exposure.

On the other hand, our study has several limitations. The relatively small sample size may limit the power of detecting a significant mediation effect. Measurement errors could be introduced by using urinary metals concentrations to assess metals exposure, as there might be individual variability of excretion of metals throughout the day. As there might be healthy worker effects if welders who participated in this study were healthier than those who left earlier due to cardiovascular diseases before the recruitment, our findings might not be generalized to the general exposed population.

Conclusion

In the current study, we observed significant direct associations between airborne metals exposure and impaired cardiac autonomic responses. Our results suggest that airborne metals exposure may affect both cardiac acceleration and deceleration capacities. The indirect effects through inflammation pathway were not statistically significant and there was no evidence suggesting inflammatory responses may mediate the effects of airborne metals exposure on cardiac autonomic changes. However, future studies in a larger population with appropriate cognizance of the exposure-mediator-response time course may be crucial for illustration of their causal relationships.

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REFERENCES


CHAPTER 4
An Epigenome-Wide Association Analysis of Cardiac Autonomic Responses among a Population of Welders.

Jinming Zhang¹
Zhonghua Liu³
Jennifer M. Cavallari⁴
Shona C. Fang⁵
Marc G. Weisskopf⁶,²
Xihong Lin³
Murray A. Mittleman²,⁶
David C. Christiani¹,²,⁷
*Corresponding author

Affiliations:

1. Department of Environmental Health, Harvard T. H. Chan School of Public Health, Boston, USA
2. Department of Epidemiology, Harvard T. H. Chan School of Public Health, Boston, USA
3. Department of Biostatistics, Harvard T. H. Chan School of Public Health, Boston, USA
4. Department of Community Medicine and Health Care, University of Connecticut Health Center, Farmington, USA
5. Department of Epidemiology, New England Research Institute, Watertown, USA
6. Cardiovascular Epidemiology Research Unit, Beth Israel Deaconess Medical Center/Harvard Medical School, Boston, USA
7. Pulmonary and Critical Care Division, Massachusetts General Hospital/Harvard Medical School, Boston, USA

Keywords: Deceleration, Acceleration, Heart Rate, Epigenetics, EWAS
Abstract

DNA methylation is one of the potential epigenetic mechanisms associated with various adverse cardiovascular effects, yet its association with cardiac autonomic dysfunction in particular is unknown. In the current study, we aimed to identify epigenetic variants associated with alterations in cardiac autonomic responses. We examined DNA methylation levels at more than 472,506 CpG sites through the Illumina Infinium HumanMethylation450 BeadChip assay. Additionally, cardiac autonomic responses were measured with two novel markers - acceleration capacity (AC) and deceleration capacity (DC). We conducted separate linear mixed models to examine associations of DNA methylation levels at each CpG site with AC and DC. We identified one CpG site (cg26938314) located in GPR133 gene which was negatively associated with DC values after multiple testing corrections through false discovery rate (FDR). Our study suggests that up-regulation of GPR133 expression is associated with impaired cardiac autonomic responses. Findings from the current study need to be replicated in future research in larger populations.
INTRODUCTION

Despite the fact that numerous therapies have been developed in cardiovascular medicine, cardiovascular disease (CVD) remains one of the leading causes of morbidity and mortality worldwide [1-3]. Understanding the biological mechanisms and predictors of CVD may have important implications in prevention and treatment of this public health concern. The cardiac autonomic nervous system consists of two major branches- the sympathetic system and the parasympathetic system and it is essential in modulation of cardiac electrophysiology [4]. Numerous studies have documented the evidence that the failure of parasympathetic control, which is often measured by conventional heart rate variability (HRV) parameters [5-6], has been a strong predictor of cardiovascular mortality in both high risk and low risk populations [7-8]. The associations of reduced HRV with various environmental and occupational pollutants, such as particulate matter air pollution with aerodynamic diameter <2.5µm (PM2.5) [9-10], ozone [11] and heavy metals [12], have been well established in previous epidemiological studies. However, the biological mechanisms by which these pollutants trigger cardiac autonomic responses remain unclear.

Few genetic variants associated with cardiac autonomic responses have been identified through genome-wide association studies (GWAS)[13-14]. However, studies also suggested that epigenetic regulation, such as DNA methylation, may also mediate the cardiac autonomic effects of environmental pollutants exposure. Air pollution studies have consistently reported the association of PM2.5 exposure with gene-specific methylation (e.g. iNOS gene) [15-16] as well as HRV changes. To date, there are no epigenetic studies of the association between DNA methylation and cardiac autonomic responses. Understanding of this relationship may elucidate the potential biological pathway of cardiac autonomic responses, which may further have implications for management and therapies for cardiovascular disease.

Therefore, we conducted an epigenome-wide association study (EWAS) to identify epigenetic variants that were associated with exposure-induced cardiac autonomic responses. Two novel markers-
acceleration capacity (AC) and deceleration capacity (DC) were used as indices of cardiac autonomic responses.

METHODS

Study population

The Harvard Boilermakers Study is a prospective cohort study conducted among a population of welders from a local boilermaker union in Quincy, MA. These welders primarily assemble or weld boilers in large power plants. In the current study, participants included 75 welders recruited from 6 sampling occasions in January 2003, January 2004, June 2010, January 2011, June 2011 and June 2012. Workers were allowed to participate multiple times within the 6 sampling occasions. The inclusion criteria were: a) male welders ≥ 18 years of age; b) unionized welders including both apprentice and journeyman; c) contributed blood and ECG samples at least once. The exclusion criteria were self-reported physician diagnosed cardiovascular disease prior participation.

During each sampling occasion, participants were monitored at a union welding school on an approximate 6-hour workday. Their major occupational activities included welding, grinding and cutting tasks. We collected urine, blood and resting ECG recordings at both prior- (baseline) and post- work. We also collected self-administrated questionnaires including age, height, weight, current smoking status and/or smoking history, medical history and medication use during the past six months from each participant at baseline, with this information updated at each sampling occasion. The Harvard T. H. Chan School of Public Health Institutional Review Board reviewed and approved the study protocol and we obtained a written informed consent from each participant at each sampling occasion.

Electrocardiographic recording and sample analysis

During each sampling occasion, participants were fitted with a 7-lead ambulatory electrocardiogram (ECG) Holter monitor. We collected 12-minute resting ECG recordings from each participant at both prior and post work. During the 12-minute resting period, participants were asked to remain seated and
quiet; walking, talking or eating was not allowed, as AC and DC are sensitive to these activities. The ECGs samples were then sent to the Cardiovascular Epidemiology Research Unit of Beth Israel Deaconess Medical Center (Boston, Massachusetts, USA), where trained technicians blinded to exposure status processed and analyzed these samples. The methods of processing ECGs samples for AC and DC analysis have been discussed in our previous study [17]. For ECGs sample analysis, the first two minutes of the 12-minutes resting ECG recordings were discarded to allow for acclimation. AC and DC quantities were computed and summarized every 5 minutes through the PRSA method, as described by Bauer et al. [18]. We collected a total of 208 5-minute AC or DC values.

**DNA methylation profiling and data quality control**

We collected whole blood samples (N=208) from each participant at both prior and post work through venous phlebotomy in EDTA tubes. Plasma was extracted and blood pellets were bisulfite-converted. DNA methylation levels of the entire genome that covers more than 480,000 CG dinucleotide (CpG) sites were determined through Infinium HumanMethylation450 BeadChip assay (Illumina, Inc.) following the Infinium HD Methylation Assay protocol guide. The BeadChips were scanned using the Illumina iScan and raw data was imported into GenomeStudio where image intensities were extracted. A methylation score (β value) was quantified on a scale from 0 to 1 to represent the percentage of methylated signal at each CpG site, such that a value of 0 represents fully unmethylated signal whereas a value of 1 represents fully methylated signal.

Following background subtraction and dye bias adjustment, both sample-level and probe-level quality control procedures were performed. Samples with detection p value > 0.05 in more than 5% probes were excluded. We omitted probes with detection p-value > 0.05 in more than 5% samples or probes with very low variation (CV<5%). We also excluded sex chromosomes and SNP associated probes. Beta-mixture quantile normalization (BMIQ) [19] was conducted for probe design bias correction. The β values were normalized with functional normalization and batch effect adjustment was performed with ComBat [20]. In total, there were 472,506 CpG sites included in the final association analyses.
Statistical analysis

In the current study, we conducted an Epigenome-Wide Association analysis and we fitted separate linear mixed effects models to examine whether DNA methylation at each CpG site was associated with AC or DC. A number of covariates including age (continuous in years), body mass index (BMI; continuous weight in kilograms divided by height squared in meters), current smoking status (non-smoker, former smoker or current smoker) and time (baseline or post-work) were adjusted for in all models as they have been suggested as potential confounders. In addition, all models were adjusted for a variable “time in day”, which reflects the time when blood and ECGs samples were collected, to account for the potential circadian variations of AC and DC. The linear mixed effects models with subject-specific intercepts regressing AC or DC ($Y_{ij}$) on each methylation site were fitted as follows:

$$Y_{ij} = \gamma_0 + \gamma_1 \text{CpG}_{ij} + \gamma_2 \text{BMI}_{ij} + \gamma_3 \text{Time in day}_{ij} + \gamma_4 \text{smoking status}_{ij} + \gamma_5 \text{Time}_{ij} + \gamma_6 \text{Age}_{ij} + b_i + \epsilon_{ij}$$

Where $\epsilon_{ij} \sim N(0, \sigma^2)$ and $b_i \sim N(0, \sigma_b^2)$. To correct for multiple comparisons, we computed the False Discovery Rate (FDR) adjusted p value (q value) with the R package. We considered an FDR q value $\leq 0.1$ as statistically significant. Additionally, the Bonferroni correction, a more stringent criterion assuming independence of all tests, was applied and a p value $\leq 1.06 \times 10^{-7}$ was defined as the epigenome-wide significance threshold.

We fitted the “Q-Q plot” to visualize the expected distribution of test statistics of the association analyses across the CpG sites (X-axis) versus the observed values (Y-axis). The Manhattan plots of AC and DC analyses were also fitted with the R package “qqman”.

In a sensitivity analysis, we omitted 5 welders who were not Caucasian. All analyses were performed with R 3.2.2 (R Core Team 2015).

RESULTS
Characteristics of the study population are shown in Table 1.3. There were 75 male welders included in the final analysis. The majority of them were Caucasian (93.3%) with the average of age being 41.6 years (ranged 21.6-71.2). Close to 40% were current smokers and average BMI was 28.7 kg/m². The baseline DC and AC values were 7.64 (SD=3.64) ms and -6.42 (SD=4.05) ms, respectively.

Table 1.3: Demographics of study populations (N=75).

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>N(%) or Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>75(100)</td>
</tr>
<tr>
<td>Age(years)</td>
<td>41.6(12.8)</td>
</tr>
<tr>
<td>Range</td>
<td>21.6-71.2</td>
</tr>
<tr>
<td>BMI(kg/m²)</td>
<td>28.7(5.3)</td>
</tr>
<tr>
<td>Race</td>
<td></td>
</tr>
<tr>
<td>Caucasian</td>
<td>70(93.3)</td>
</tr>
<tr>
<td>African American</td>
<td>3(4.0)</td>
</tr>
<tr>
<td>Hispanic</td>
<td>1(1.3)</td>
</tr>
<tr>
<td>Asian</td>
<td>1(1.3)</td>
</tr>
<tr>
<td>Current smoker</td>
<td>28(37.3)</td>
</tr>
<tr>
<td>Acceleration Capacity(ms)</td>
<td>-6.42(4.05)</td>
</tr>
<tr>
<td>Deceleration Capacity(ms)</td>
<td>7.64(3.64)</td>
</tr>
</tbody>
</table>

We examined the associations of methylation levels at 472506 CpG sites in the whole-genome with AC or DC, while adjusting for age, BMI, smoking status, time and time in day. Figure 1.2 shows the Quantile-Quantile (QQ) plots for AC or DC analyses. For CpGs located within genes, we also reported the gene names.

Figure 1.2: Quantile-Quantile (QQ) plot*

- a). DC*
- b). AC*

*The observed p-values (y-axis) were plotted against the expected p-values under the null hypothesis (x-axis). The red diagonal line denotes the pattern under null hypothesis.

*Figure 1a). Quantile-quantile plot from EWAS of DC analysis.
*Figure 1b). Quantile-quantile plot from EWAS of AC analysis.
For DC analyses, only one CpG site (cg26938314) located in the GPR133 gene on chromosome 12 (Figure 2.2) reached genome-wide significance when using either a threshold of FDR q-value ≤0.1 or Bonferroni correction (Table 2.3). For CpG sites that yielded p values < 1E-6, an additional CpG site (cg12400881) located in PPL gene on chromosome 16 was identified, although it was not statistically significant. We observed a significantly negative association between methylation levels at cg26938314 with DC (β=-0.30, SE=0.04; p=2.26E-08). For AC analyses, two CpG sites (cg15275103 and cg13759632) were genome-wide significant with FDR q values ≤0.1 (Table 3.3). However, they are not linked with any known functional genes. In the sensitivity analysis, after excluding 5 subjects who were non-white, the statistically significant association between methylation levels at cg26938314 with DC persisted, the estimated coefficients and p value (β=-0.32, SE=0.04; p=1.98E-08) was comparable with those in the main analysis.
Table 2.3: Summaries of top-ranking CpG sites associated with DC\textsuperscript{a}.

<table>
<thead>
<tr>
<th>Name</th>
<th>CHR</th>
<th>Strand</th>
<th>Gene name</th>
<th>Location</th>
<th>Coefficients</th>
<th>SE</th>
<th>P value</th>
<th>FDR q value\textsuperscript{b}</th>
<th>Bonferroni</th>
</tr>
</thead>
<tbody>
<tr>
<td>cg26938314</td>
<td>12</td>
<td>F</td>
<td>GPR133</td>
<td>Body</td>
<td>-0.30</td>
<td>0.04</td>
<td>2.26E-08</td>
<td>0.011</td>
<td>1.03E-07</td>
</tr>
<tr>
<td>cg12400881</td>
<td>16</td>
<td>R</td>
<td>PPL;PPL</td>
<td>1stExon;5'UTR</td>
<td>0.47</td>
<td>0.10</td>
<td>8.69E-07</td>
<td>0.21</td>
<td>1.03E-07</td>
</tr>
<tr>
<td>cg08432559</td>
<td>11</td>
<td>F</td>
<td></td>
<td></td>
<td>-0.27</td>
<td>0.06</td>
<td>9.09E-06</td>
<td>0.683</td>
<td>1.03E-07</td>
</tr>
<tr>
<td>cg15132372</td>
<td>11</td>
<td>R</td>
<td></td>
<td></td>
<td>0.24</td>
<td>0.05</td>
<td>9.24E-06</td>
<td>0.683</td>
<td>1.03E-07</td>
</tr>
<tr>
<td>cg12044338</td>
<td>1</td>
<td>F</td>
<td>AQP10</td>
<td>TSS1500</td>
<td>0.47</td>
<td>0.10</td>
<td>8.69E-07</td>
<td>0.21</td>
<td>1.03E-07</td>
</tr>
<tr>
<td>cg03030958</td>
<td>13</td>
<td>F</td>
<td></td>
<td></td>
<td>0.26</td>
<td>0.05</td>
<td>9.09E-06</td>
<td>0.683</td>
<td>1.03E-07</td>
</tr>
<tr>
<td>cg00060975</td>
<td>4</td>
<td>R</td>
<td></td>
<td></td>
<td>-0.27</td>
<td>0.06</td>
<td>9.24E-06</td>
<td>0.683</td>
<td>1.03E-07</td>
</tr>
<tr>
<td>cg19147912</td>
<td>11</td>
<td>R</td>
<td></td>
<td></td>
<td>-0.27</td>
<td>0.06</td>
<td>9.24E-06</td>
<td>0.683</td>
<td>1.03E-07</td>
</tr>
<tr>
<td>cg11633204</td>
<td>8</td>
<td>R</td>
<td></td>
<td></td>
<td>0.18</td>
<td>0.04</td>
<td>2.26E-08</td>
<td>0.011</td>
<td>1.03E-07</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Adjusted for age, BMI, smoking status, time and time in day.

\textsuperscript{b}FDR q value <0.1 was considered as the genome-wide significance threshold.

Table 3.3: Summaries of top-ranking CpG sites associated with AC\textsuperscript{a}.

<table>
<thead>
<tr>
<th>Name</th>
<th>CHR</th>
<th>Strand</th>
<th>Gene name</th>
<th>Location</th>
<th>Coefficients</th>
<th>SE</th>
<th>P value</th>
<th>FDR q value\textsuperscript{b}</th>
<th>Bonferroni</th>
</tr>
</thead>
<tbody>
<tr>
<td>cg15275103</td>
<td>10</td>
<td>R</td>
<td></td>
<td></td>
<td>-0.29</td>
<td>0.05</td>
<td>4.01E-07</td>
<td>0.092</td>
<td>1.03E-07</td>
</tr>
<tr>
<td>cg13759632</td>
<td>11</td>
<td>F</td>
<td></td>
<td></td>
<td>-0.25</td>
<td>0.04</td>
<td>4.07E-07</td>
<td>0.098</td>
<td>1.03E-07</td>
</tr>
<tr>
<td>cg02030958</td>
<td>13</td>
<td>F</td>
<td>NM_006651</td>
<td>Body</td>
<td>0.26</td>
<td>0.05</td>
<td>5.79E-06</td>
<td>0.935</td>
<td>1.03E-07</td>
</tr>
<tr>
<td>cg18758585</td>
<td>1</td>
<td>F</td>
<td></td>
<td></td>
<td>-0.58</td>
<td>0.13</td>
<td>1.99E-05</td>
<td>0.999</td>
<td>1.03E-07</td>
</tr>
<tr>
<td>cg24430034</td>
<td>13</td>
<td>R</td>
<td></td>
<td></td>
<td>0.43</td>
<td>0.09</td>
<td>1.99E-05</td>
<td>0.999</td>
<td>1.03E-07</td>
</tr>
<tr>
<td>cg00060975</td>
<td>4</td>
<td>R</td>
<td></td>
<td></td>
<td>-0.27</td>
<td>0.06</td>
<td>2.18E-05</td>
<td>0.999</td>
<td>1.03E-07</td>
</tr>
<tr>
<td>cg02704931</td>
<td>15</td>
<td>R</td>
<td>NM_001018090</td>
<td>Body</td>
<td>-1.00</td>
<td>0.22</td>
<td>2.27E-05</td>
<td>0.999</td>
<td>1.03E-07</td>
</tr>
<tr>
<td>cg19147912</td>
<td>17</td>
<td>R</td>
<td>NM_030665</td>
<td>5'UTR</td>
<td>-0.43</td>
<td>0.09</td>
<td>2.69E-05</td>
<td>0.999</td>
<td>1.03E-07</td>
</tr>
<tr>
<td>cg11633204</td>
<td>8</td>
<td>R</td>
<td>NM_00637</td>
<td>TSS1500</td>
<td>0.18</td>
<td>0.04</td>
<td>2.71E-05</td>
<td>0.999</td>
<td>1.03E-07</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Adjusted for age, BMI, smoking status, time and time in day.

\textsuperscript{b}FDR q value <0.1 was considered as the genome-wide significance threshold.
Discussion

In the current study, we aimed to identify CpGs locations at which the methylation levels were associated with cardiac autonomic changes among a population of welders. For DC, only one CpG site was genome-wide significant after correction for multiple testing (FDR q<0.1) and the significance persisted after Bonferroni correction, a stricter criterion. There was a significantly negative association between methylation at cg26938314 and DC values. Annotation analysis indicated that this significant CpG site was located in gene body of GPR133 gene.

The role of DNA methylation in regulation of gene-expression may vary depending upon different genomic contexts [21]. DNA methylation in the promoter sequences is known to down-regulate gene expression [22], whereas methylation in the gene body is often positively correlated with gene expression [23]. Decreased DC, which reflects cardiac autonomic imbalance between sympathetic and parasympathetic controls, has been identified as a strong predictor of cardiovascular mortality among patients after myocardial infarction [18,24]. Therefore, our study indicated that GPR133 gene overexpression due to epigenetic up-regulation might be associated with cardiac autonomic dysfunction, although the underlying biological mechanisms remain to be elucidated.

GPR133 is a protein-coding gene for adhesion G-protein-coupled receptor (aGPCR), which is commonly characterized by long extracellular N termini that are composed of a seven transmembrane spanning domain [25]. It is primarily expressed in the central nervous system (CNS) [26] as well as heart, such as ventricles, atria and septal tissues. Bohnekamp et al. [27] have reported a concentration– dependent relationship between GPR133 and intracellular cyclic adenosine 3',5'-cyclic adenosine monophosphate (cAMP) levels, suggesting that GPR133 may be coupled to the Gs protein and stimulate G protein cascades through activation of adenylate cyclase. The activation of adenylate cyclase is associated with various cardiovascular effects including modulation of heart rate. In the heart, adenylyl cyclase may activate the production of intracellular cyclic adenosine 3',5'-cyclic adenosine monophosphate (cAMP) [28-29], which serves as the second messenger that further binds to protein kinase A and modulate
cardiac contractility [30]. Meanwhile, the rate of generation of action potential in the sinoatrial (SA) node may be dependent on increased intracellular cAMP levels [31].

Both toxicological studies and genome-wide association studies (GWAS) have documented associations of several subtypes of GPCRs with adverse cardiovascular effects, such as hyperproliferative vascular malformations (GPR124) [32], myocardial wall thinning (GPR126) [33], stroke (CELSR1) [34] and myocardial infarction (CELSR2) [35]. To date, several GWAS studies have been conducted to explore genetic contributions to cardiac autonomic responses. Arking et al. [14] identified the NOS1AP (CAPON) gene associated with the electrocardiographic (ECG) QT interval variations among participants from the KORA cohort in Germany. Marroni et al. [13] observed similar findings and they additionally identified the associations of variants in GPR133 gene with the electrocardiographic (ECG) RR interval alterations. Newton-Cheh et al. [36] examined 70,987 common genetic variants and six conventional heart rate variability (HRV) parameters among 1345 participants from Framingham Heart Study Original and Offspring cohort, there was no genomic hit that yielded a genome-wide significance. Our study, however, expands the literature with Epigenome-wide association studies (EWAS) and suggests that epigenetic regulations of GPR133 gene may play a role in heart rate modulations.

The second-ranked CpG site associated with DC is located in the PPL (periplakin) gene, although it might not reach genome wide significance due to small sample size of the study. PPL gene is a protein-coding gene responsible for keratinocyte intercellular adhesion as well as tissues integrity [37]. Decreased periplakin expression has been associated with urothelial carcinoma of the urinary bladder (UCB) [38] and serum periplakin has been suggested as a biomarker for UCB [39]. However, there was no evidence suggesting the role of periplakin in cardiovascular disease from previous studies. Its association with cardiac autonomic responses may warrant further research.

To the best of our knowledge, this is the first epigenome-wide association study investigating epigenetic variants associated with cardiac autonomic responses. GPCRs are one of most studied receptor families that are used as pharmacological targets [40]. In the heart, the adrenergic GPCR signaling pathways are
one of the major targets for pharmaceuticals for treatment of cardiovascular disease. For example, beta-blockers have been extensively used for management of arrhythmia, hypertension and chronic heart failure [41]. Our study indicated a potential role of GPR133 in cardiac autonomic dysfunction primarily through affecting the deceleration capacity of heart rate. Future studies may investigate the association of GPR133 gene with various cardiovascular diseases, such as abnormal heart rhythms and heart failure.

Our study is limited by the relatively small sample size and lack of independent replication. Therefore, our findings need to be confirmed in future studies among larger populations. In addition, DNA methylation was measured using the total white blood cells (WBC) and it is known that different subtypes of WBC might be associated with differential methylation signatures [42]. Previous studies have reported that activation of sympathetic effects was associated with alterations in the subset distribution of lymphocytes [43-44]. Therefore, “differences in WBC subtypes” may have been a confounder in this study.

Additionally, previous epidemiological studies have reported the strong associations of acute and chronic PM2.5 exposure with DNA methylation of several genes as well as cardiac autonomic responses. For example, both short term and long term particulate matter exposure from welding fumes were significantly associated with AC and DC as well as conventional HRV parameters changes among this welder cohort. PM2.5 exposure has also been negatively associated with gene-specific (iNOS) methylation in a population of boilermaker construction workers as well as elderly men from the VA Normative Aging Study. Hence, we cannot preclude the potential confounding effects of PM2.5 due to lack of data in this study. Meanwhile, there might be other potential confounders in the occupational settings, such as heavy metals and psychological factors, in addition to PM2.5. Taken together, the association between DNA methylation and DC needs to be verified in the future study.

In conclusion, results of our study indicate that up-regulation of the GPR133 gene may play a role in cardiac autonomic dysfunction. However, we cannot preclude chance or bias due to lack of replication,
small sample size and potential confounding by pollutants exposure. Hence, our preliminary findings need to be confirmed in future research.

ACKNOWLEDGEMENTS

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COMPETING INTERESTS

The authors declare that they have no competing interests.

REFERENCES


CHAPTER 5. CONCLUSIONS
SUMMARY AND CONCLUSIONS

This dissertation expands current knowledge on the cardiac autonomic effects of metals exposure as well as understanding on potential biological mechanisms of these effects. In summary, we observed negative associations of exposure to mercury and chromium with DC and positive associations of exposure to mercury, chromium and manganese with AC. In addition, metals exposures were directly associated with AC or DC after accounting for inflammatory responses. However, there was no evidence suggesting inflammation might mediate the effects of metals exposure on cardiac autonomic responses. While several limitations of the current mediation analysis were discussed, it has been suggested that future studies in larger populations with appropriate time course of sampling framework are warranted to determine the underlying causal relationships. Furthermore, we identified an epigenetic variant located in gene body of GPR133 associated with DC, suggesting the regulation of GPR133 might play a role in modulation of heart rate. Overall, due to small sample size and lack of replication study, findings from the current project may warrant future research.