

# EPIGENETIC CHANGES AND CARDIOVASCULAR RISKS AMONG WORKERS OCCUPATIONALLY EXPOSED TO IRON AND ZINC

By

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## Abstract

**Introduction:** Several metals such as Iron and Zinc were found at high concentration in foundry particulate matter and demonstrated to have pro-coagulant effects, these effects may occur through epigenetic changes of pro-inflammatory genes. So, Iron and Steel workers are at a high risk for cardiovascular diseases. **Aim of work:** Evaluation of the risk of thrombosis and cardiovascular diseases among individuals occupationally exposed to Iron and Zinc. **Materials and methods:** A descriptive cross sectional comparative study was performed on 60 persons working in Iron and Steel factory and 60 subjects used as control group with no history of occupational exposure to Fe or Zinc. All study population was subjected to personal interview with specially designed questionnaire. Serum Iron and Zinc levels together with Endogenous thrombin potential (ETP) test were measured. Assessment of methylated DNA of Endothelin-1 (EDN1) and Nitric-oxide synthase-3 (NOS3) genes were performed. **Results:** the current study showed highly statistically significant increase of serum Iron, Zinc levels and percentage of ETP among exposed group when compared to the control with Mean  $\pm$  SD 125.6 $\pm$ 22.9, 110.9 $\pm$ 19.2, 168 $\pm$ 0.5 vs 63.6 $\pm$ 15.3, 42.5 $\pm$ 7.2, and 102.22 $\pm$ 12.36 respectively. There was also a significant decrease of methylated DNA of EDN1 and NOS3 genes among exposed group with Mean  $\pm$  SD 0.71 $\pm$ 0.3, 0.75 $\pm$ 0.3 versus 1.51 $\pm$ 1.01, 4.09 $\pm$ 0.68 respectively among the control group. Serum levels of Iron and Zinc and percentage of ETP were negatively correlated with methylated DNA of

NOS3 and EDN1 genes  $r = -0.304$ ,  $r = -0.450$ ,  $r = -0.259$ ,  $r = -0.787$ ,  $r = -0.866$  and  $r = -0.618$  respectively ( $p$  value  $< 0.005$ ), while positive correlation were detected between Iron and Zinc levels with ETP ( $r = 0.692$  and  $r = 0.625$ ). It was found that Iron, Zinc and NOS3 are determinants for EDN1 while the latter is the only predictor for NOS3. **Conclusion:** The risk of thrombosis and cardiovascular diseases were related to occupational exposure to high levels of Iron and Zinc. So, regular wearing of good quality's personal protective equipment, especially masks and gloves, is highly recommended to decrease exposure to these metals.

**Key words:** Iron, Zinc, Methylated DNA, Endogenous thrombin potential (ETP) and Nitric-oxide synthase-3 (NOS3) genes.

### Introduction

Iron and steel industry include various materials and processes. It produces shaped castings by passing through pattern-making, molding and core-making, melting of metal ingots and scrap, pouring, shake-out and finally cast fettling. Furnace tenders, smelters, casters, ladle-men, pourers and crane drivers were established as the main sites for workers exposure by IARC, 1984. Alley et al., 2009 and Fang et al., 2009 demonstrated that the indoor particulate matter (PM) concentration in working environment is higher than outdoor concentrations. Even in modern steel plants, the workers are exposed to inhalable PM concentrations higher than those measured in ambient outdoor air with a large proportion of potentially toxic metal components. Some studies suggested a relationship between metal rich PM and the thrombotic effects which contribute to cardiovascular

mortality and morbidity (Sarah and Mark, 2018 and Yan Li et al., 2019). Some of these metals are Iron and Zinc which are found at high concentrations in foundry environment PM and have a great toxicity (Arslan et al., 2010). They produce several pro-thrombotic modifications through alteration of clotting factors activity, increased platelet aggregation, reduced clotting time, and higher expression of pro-coagulant genes and proteins (Arslan et al., 2010; and Cantone et al., 2015).

Nitric oxide (NO) and endothelin-1 (ET-1) are natural counterparts in vascular function. Nitric oxide produces vasodilatation by regulating vascular tone and inhibits the synthesis and the vasoconstrictive effects of endothelin-1. It was suggested by recent studies that endothelin-1 is important for nitric oxide signaling. As any increase in endothelin-1 leads to production of

nitric oxide to counteract its effects. An imbalance between these two mediators leads to endothelial dysfunction and progression of vascular disease. In case of diminished nitric oxide bioavailability, the unbalanced effects ET-1 result in vasoconstriction that end in vascular remodeling and dysfunction (Bourque et al., 2011).

These mediators are encoded by Nitric-oxide synthase-3(NOS3) and Endothelin-1 (EDN1) genes which are considered as pro-coagulant genes and have pro-inflammatory activity in human body (Browatzki et al., 2005 and Nearmeen et al., 2017). Transcriptions of these genes are controlled by environmentally-sensitive epigenetic mechanism known as DNA methylation (Baccarelli et al., 2010).

Methylated DNA usually marks genes and become silence or inactive, while hypomethylated genes are easy to get to transcription and become active or ready to be activated (Chan et al., 2004, Vallender and Lahn, 2006).

Thrombin is a key enzyme in clot formation and describes the overall coagulability. It decreases in patients treated with anticoagulants and increases in patients with thrombophilia (Kakkar et al., 2002), recently, it has

been correlated with high risk of venous thromboembolism (Tripodi et al., 2007).

The time course of formation and decay of thrombin is represented by Endogenous Thrombin Potential (ETP) test and is used as a global coagulation assessment (Siegemund et al., 2004).

### **Aim of work**

Evaluation of the risk of thrombosis and cardiovascular diseases among individuals occupationally exposed to Iron and Zinc.

### **Materials and methods**

**Study design:** This is a comparative cross sectional study.

**Place and duration of the study:** The study was conducted in iron and steel factory at Helwan district, Cairo, Egypt during the period of October 2017 to January 2018.

**Study sample:** All workers at the Iron furnaces in the studied factory were invited to participate in the study after illustrating the aim of the study and the importance of sharing in it. They were continuously employed at the factory for (8hrs/day, 6 days/week) for duration of 5 - 37 years. After exclusion of non-responders, and application of exclusion criteria (persons who

were with a history of blood diseases or under medical treatment affecting blood coagulation), the recruited workers were 60. The control group was composed of 60 participants from the administrative departments at the same factory, with no history of exposure to harmful metals, and was matched with the exposed group for age, sex, lifestyle and economic status.

### Study methods:

1- Each participant in the study was subjected to a specially **designed detailed questionnaire** including personal, occupational, cardiovascular, medical, present, past and family histories and history of use of personal protective equipments.

### 2- General and local clinical

**examination** was carried out.

### 3- Laboratory investigations:

10 ml of venous blood were drawn under aseptic conditions for investigations of:

#### A- DNA methylation analysis

DNA extraction and bisulfite treatments (Qiagen) were performed. The amplicons in promoter CpG-rich areas were selected using Applied Biosystem Software (Applied Biosystem Software Foster city, USA) for assessment of methylated NOS3 and EDN1 genes and amplified the sequences as shown in Table 1 (Primer concentrations and sequences, and PCR cycling conditions are shown in table 2 and 3 respectively). For more reliability, each sample was tested two times.

**Table 1: Primers for DNA methylation analysis.**

Sequence ID	Primer sequence (5' to 3')	Gene bank accession number
<b>NOS3</b>	Forward: TGTAGTTTTAGGGTTTTGTTGGA Reverse: FAM-CCCCTATCCCATACACAAT	AF387340.1
<b>EDN1</b>	Forward: TTGTTTGGGGTTGGAATAAAGT Reverse: FAM-ATCCTTCAACCCAATACCCTTTT	AC109333.28

**Table 2: The reaction mixture was prepared according to the following table:**

q PCR reaction mix	Amount (1 Test =20µl)
2x RealMOD™ Real-time PCR Master mix Solution	10 µl
Primers (Forward+ Reverse)	2 µl
Template methylated DNA	5µl
RNase free water	3µl

**Table 3: PCR thermal profile**

Stages	Temperature	Time	Number of cycle
Stage 1. Initial Denaturation	95°C	5 min	1
Stage 2. PCR Denaturation Anneal/Extend	92°C 60°C	15 sec. 60 sec.	45

**B-Iron status:**

Measurement of Iron level (µg/dl) for each patient was done using biochemical tests including colorimetric kit (StanbioLabrotary, Boerne, TX USA).

**C-Zinc level**

Atomic Absorption Spectrometry (AAS) (Perkin Elimer, Germany) was used to measure the Zinc level. An aliquots (0.5 mL) of plasma were mixed with 5 mL of the digestion solution

(HClO<sub>4</sub>: HNO<sub>3</sub>; 3:7). The digested samples were heated until dry and stored at room temperature for 4–6 h. The dried samples were re-dissolved in 5 ml of 1% HNO<sub>3</sub> prior to AAS analysis. The wavelength of 248.3 for AAS analysis was used for Zn (Correia et al., 2002).

**D-Endogenous thrombin potential (ETP)**

Blood for ETP testing was drawn into vacuum tubes containing 0.5 ml trisodium citrate at a ratio of 5:1 (blood/

anticoagulant) and centrifuged for 10 minutes at 2,500g at room (controlled) temperature and plasma was aspirated in plastic tubes to measure thrombin in the presence of thrombomodulin to mimic much more closely the conditions operating in vivo (Bonzini et al., 2010).

### **Consent**

The purpose of the study was declared to all participants. A verbal consent was taken from all workers who agreed to participate in the research work. All personal information about the study participants was kept confidential.

### **Ethical approval**

Approval of the study was taken from the internal Ethical Committee of Occupational and Environmental Medicine department, Faculty of Medicine, Cairo University. As well, approval from the factory manager was obtained.

### **Data management**

The statistical package SPSS (Statistical Package for the Social Sciences) version 25 was used for data entering and coding. Quantitative data was summarized using mean, standard deviation, median, minimum and maximum while frequency (count) and relative frequency (percentage) were used for categorical data. The independent t test was used for comparison between quantitative variables (Chan, 2003a) and Chi square ( $\chi^2$ ) test for comparing categorical data. Exact test was used instead when the expected frequency is less than 5 (Chan, 2003b). Pearson's correlation coefficient was used for correlations between quantitative variables (Chan, 2003c). Linear regression analysis was done to detect independent predictors of END-1, NOS3 and ETP (Chan, 2004). P-values less than 0.05 were considered as statistically significant.

## Results

**Table (1): Demographic characteristics and associated symptoms of the studied group.**

	Exposed (No=60)		Non exposed (No=60)		p value
Age# (Mean± SD)	46.3±9.4		43.5±8.58		0.08
Smoking index£#					
Minimum	0		0		0.02
Maximum	680		400		
Median	26		0		
Characteristics	No	%	No	%	p value
Cough ##					
NO	46	76.7	54	90	<b>0.05*</b>
Yes	14	23.3	6	10	
Expectoration ##					
NO	46	76.7	54	90	<b>0.05*</b>
Yes	14	23.3	6	10	
Dyspnea ##					
NO	45	75	60	100	<b>&lt;0.001**</b>
Yes	15	25	0	0	
Wheezes ##					
NO	50	83.3	60	100	<b>0.001**</b>
Yes	10	16.7	0	0	
Diabetes ##					
NO	53	89.8	55	91.7	0.7
Yes	6	10.2	5	8.3	
HTN ##					
NO	59	98.3	54	90	0.11
Yes	1	1.7	6	10	
Skin affection ##					
NO	59	98.3	60	100	0.99
Yes	1	1.7	0	0	

#: Independent t test (Mean ± SD)

## Chi-square (X<sup>2</sup>)

\*: Statistically significant

\*\* : Highly statistically significant

£: Smoking index: number of cig. /day × duration of smoking in years.

Table (1) showed highly statistically significant difference between exposed and control groups as regards dyspnea and wheezes (p value <0.001), and statically significant difference between both groups as regards cough and expectoration (p value =0.05) while no significant differences were found for other symptoms.

**Table (2): Mean  $\pm$  SD of serum iron and zinc levels, methylated DNA of Nitric-oxide synthase-3 (NOS3), and Endothelin-1 (EDN1) genes and percentage of Endogenous Thrombin Potential (ETP) using independent t test .**

Parameters	Exposed (No=60) Mean $\pm$ SD	Non-exposed (No=60) Mean $\pm$ SD	p value
Iron ( $\mu$ g/dl)	125.6 $\pm$ 22.9	63.6 $\pm$ 15.3	<0.001**
Zinc ( $\mu$ Mol/L)	110.9 $\pm$ 19.2	42.5 $\pm$ 7.2	<0.001**
NOS3	0.71 $\pm$ 0.3	1.51 $\pm$ 1.01	<0.001**
END-1	0.75 $\pm$ 0.3	4.09 $\pm$ 0.68	<0.001**
ETP %	168 $\pm$ 0.5	102.22 $\pm$ 12.36	<0.001**

\*\* : Highly statistically significant

NOS3: Nitric-oxide synthase-3

END1: Endothelin-1

ETP: Endogenous Thrombin Potential

Table (2) showed highly statistically significant increase of serum Iron and Zinc levels with increase of Endogenous Thrombin Potential among exposed when compared to the control group. It also showed a decrease of methylated DNA of Nitric-oxide synthase-3 (NOS3), and Endothelin-1 (EDN1) genes among the exposed when compared to the control group (p < 0.001).

**Table (3): Pearson's correlation coefficient between duration of exposure, Iron, Zinc and percentage of Endogenous Thrombin Potential (ETP) with smoking index, methylated DNA of Nitric-oxide synthase-3 (NOS3), and Endothelin-1 (EDN1) genes among the exposed group (No=60).**

Parameters		Duration of exposure	Smoking index	NOS3	END1	Iron	Zinc	ETP
-Duration of exposure	R	---	0.378	-0.139	-0.143	-0.166	-0.209	-0.045
	p value		<b>0.003**</b>	0.291	0.276	0.205	0.109	0.732
-Smoking index	R	0.378	---	-0.232	-0.229	0.183	0.172	0.144
	p value	<b>0.003**</b>	---	<b>0.011*</b>	<b>0.012*</b>	<b>0.046*</b>	0.060	0.117
-Iron	R	-0.166	0.183	-0.304	-0.787	---	0.790	0.692
	p value	0.205	<b>0.046*</b>	<b>0.001**</b>	<b>0.000**</b>	---	<b>0.000**</b>	<b>0.000**</b>
-Zinc	R	-0.209	0.172	-0.450	-0.866	0.790	---	0.625
	p value	0.109	0.060	<b>0.000**</b>	<b>0.000**</b>	<b>0.000**</b>	---	<b>0.000**</b>
-NOS3	R	-0.139	-0.232	---	0.470	-0.304	-0.450	-0.259
	p value	0.291	<b>0.011*</b>	---	<b>0.000**</b>	<b>0.001**</b>	<b>0.000**</b>	<b>0.004**</b>
-ETP	R	-0.045	0.144	-0.259	-0.618	0.692	0.625	---
	p value	0.732	0.117	<b>0.004**</b>	<b>0.000**</b>	<b>0.000**</b>	<b>0.000**</b>	---

\*: Statistically significant      \*\*: Highly statistically significant      NOS3: Nitric-oxide synthase-3  
 END1: Endothelin-1      ETP: Endogenous Thrombin Potential

Table (3) showed statistically significant positive correlation between Iron and Zinc and between Iron and Zinc with ETP. While negative correlations were found with NOS3 and END1. As regards ETP, statistically significant negative correlations were found with NOS3 and END1.

**Table (4): Multiple linear regression models for detecting predictors of methylated DNA of Nitric-oxide synthase-3 (NOS3), and Endothelin-1 (EDN1) genes and percentage of Endogenous Thrombin Potential (ETP) among exposed group (No=60).**

Predictors	Parameters	$\beta$	p value
<b>Predictors for NOS3</b>			
	Duration of exposure	-0.004	0.686
	Iron	0.006	0.097
	Zinc	-0.006	0.105
	END1	0.208	<b>0.014 *</b>
	ETP	0.000	0.933
<b>Predictors of END1</b>			
	Duration of exposure	-0.006	0.178
	Iron	-0.013	<b>0.001**</b>
	Zinc	-0.027	<b>0.000**</b>
	NOS3	0.250	<b>0.014*</b>
	ETP	-0.002	0.461
<b>Predictors of ETP</b>			
	Duration of exposure	-0.231	0.471
	Iron	0.794	<b>0.006**</b>
	Zinc	0.007	0.982
	END1	0.693	0.966
	NOS3	48.189	<b>0.018*</b>

\*: Statistically significant

\*\* : Highly statistically significant

END1: Endothelin-1

NOS3: Nitric-oxide synthase-3

ETP: Endogenous Thrombin Potential

Table (4) showed that the only predictor for NOS3 is END1 while Iron, Zinc and NOS3 are predictors for END1. As for ETP, it was found that Iron and NOS3 are determinants for it ( $p < 0.01$ ).

**Table (4): Multiple linear regression models for detecting predictors of methylated DNA of Nitric-oxide synthase-3 (NOS3), and Endothelin-1 (EDN1) genes and percentage of Endogenous Thrombin Potential (ETP) among exposed group (No=60).**

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\*: Statistically significant      \*\*: Highly statistically significant      END1: Endothelin-1  
 NOS3: Nitric-oxide synthase-3      ETP: Endogenous Thrombin Potential

Table (4) showed that the only predictor for NOS3 is END1 while Iron, Zinc and NOS3 are predictors for END1. As for ETP, it was found that Iron and NOS3 are determinants for it ( $p < 0.01$ ).

## Discussion

The metal foundries are considered an important industry in most countries in the world where millions of workers are employed in it. The health risk is high for workers involved in all the processes because of emissions of harmful heavy metals at the workplace (Narjes et al., 2017).

The respiratory tract is an important route for heavy metal absorption and toxicity. Lung damage can be caused by oxidative stress, inflammation and modulation of the immune response resulting from inhalation of particulate matter (PM). Asthma, reduction in lung growth, allergic rhinitis and respiratory infections are the most frequently reported effects of metals (Fortoul et al., 2015).

The current study showed a statistically significant affection of the lung with symptoms of dyspnea and wheezes among exposed group when compared to control (Table 1). These results resembles that of Dany et al., 2017 in their study on residential air pollution and associations with wheeze and shortness of breath in adults ; data from two large European cohorts and detected a significant relationship between inhaled PM of metals and

occurrence of wheezes and shortness of breath.

Another cross-sectional study conducted by Manish et al., 2014 in an Indian Iron and Steel industry among 400 workers showed that 60% of the workers had occupational morbidities from which 20% had chronic bronchitis and 6% had bronchial asthma.

In addition to respiratory affection, the present study showed statistically significant high levels of Iron and Zinc among the exposed group when compared to the control (Table 2). This was similar to the results detected by Tarantini et al. 2013 in their study on blood Hypo-methylation of inflammatory genes mediates the effects of metal-rich airborne pollutants on blood and found that the level of Iron and Zinc were high among steel workers.

In another study at Swedish Iron foundries conducted by Håkan et al., 2019, workers who are exposed to quartz dust showed statistically significant increased levels of serum Iron as well as inflammation and coagulation markers, namely fibrinogen and factor VII (FVII).

The impact of environmental PM

exposures on health through epigenetic changes and DNA methylation is one of the most exciting areas developed in recent years. (Marczylo et al., 2016). Decrease in methylated DNA is associated with increases in gene transcription, fibrinogen protein expression and hypercoagulability state (Baccarelli et al., 2010).

The present study showed statistically significant decrease of methylated DNA of NOS3 and END 1 among the exposed workers when compared to the control and negative correlations were found between them with the blood levels of Iron and Zinc (Table 2 and 3). These agreed with Tarantini et al., 2013 results who demonstrated that exposure to particulate matters rich in metals in the workplace is associated with increase in the levels of Iron and Zinc and decrease in methylated DNA.

The association between short and long-term particulate air pollution exposure and changes in homeostasis and enhanced thrombosis were strengthened by much of the recent epidemiological research; enhanced thrombosis was potentially leading to heart attack, ischemic stroke or death (Kelly and Fussel, 2017).

Thrombin is one of the factors involved in thrombosis process. The total amount of thrombin generated in plasma can be measured by Endogenous thrombin potential (ETP) test and increased levels have been proposed as an index of hypercoagulability (Ina et al., 2018). The present study showed an increased level of ETP among exposed group relative to the control. Positive correlations were found between ETP with Iron and Zinc levels among the exposed group, while it was negatively correlated with methylated DNA of NOS3 and EDN1 genes (Table 2 and 3). These agreed with the results of Matteo et al., 2010 on workers from a steel plant in Northern Italy which detected higher metal rich PM exposure with inflammation and coagulation markers using ETP test.

As endothelin-1 is an essential component of NO signaling, this may explain why END1 is the only determinant of NOS3, while Iron, Zinc and NOS3 are predictors for END1, for ETP, it was found that Iron and NOS3 are determinants for it (Table 4). These findings were consistent with the results obtained by Bonzini et al., 2010 in their study on the effects of inhalable particulate matter on blood coagulation.

Also Sarah and Mark, 2018 in their study on ambient air pollution and thrombosis support the role of pro-thrombotic effect of occupational exposure to PM containing metals through the activation of multiple pathophysiological processes, which likely will contribute to the overall cardiovascular morbidity among workers.

### **Conclusion and recommendations:**

Iron and Steel workers are exposed to high levels of Iron and Zinc which are significant heavy metal occupational toxins, especially, within the metal foundries industry. Our study showed that there was a highly statistically significant increase of serum Iron and Zinc levels with increase of Endogenous Thrombin Potential (ETP) among exposed group, with decrease of methylated DNA of Nitric-oxide synthase-3 (NOS3), and Endothelin-1 (EDN1) genes. These results mean that Iron and Zinc produce oxidative stress stimulating transcription of endothelin-1 and nitric oxide, and imbalance between them leads to vascular dysfunction and hypercoagulability effects. So, those workers are at high risk of thrombosis and cardiovascular diseases.

Epigenetic changes and DNA

methylation are considered new methods for early detection of cardiovascular diseases and we hope to be used in periodic medical examination. So, workers need adequate protection from the vapors produced during metal working. It is in the interests of companies to provide very efficient protective measures with adequate health education program.

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### **Conflict of interest**

There is no conflict of interest.

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