

# THE RELATIONSHIP OF BODY IRON STORES AND OXIDATIVE STRESS MARKERS RELATED TO ATHEROSCLEROSIS IN WOMEN OF REPRODUCTIVE AGE

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## Abstract

**BACKGROUND:** Elevated body iron stores have been suggested to be a risk factor for cardiovascular disease (CVD). We examined whether elevated plasma ferritin concentrations as an indicator of iron stores would affect the oxidative stress markers in women of reproductive age.

**METHODS:** One hundred sixty, women, aged 20-45 years, were selected randomly. Samples had no proven heart disease. Demographic data were gathered using check lists and face-to-face interviews. We investigated body iron stores by measuring the concentrations of plasma ferritin. Furthermore, we assessed oxidative stress markers by measuring the concentrations of plasma malondialdehyde (MDA) and activities of erythrocyte cytoprotective enzymes.

**RESULTS:** Subjects in the highest tertile of plasma ferritin presented the highest levels of plasma MDA ( $p < 0.05$ ). Those in the highest tertile of plasma ferritin had also the highest mean values of CAT activity ( $p < 0.05$ ). Furthermore, Subjects in the highest tertile of plasma ferritin presented the lowest levels of CuZn-SOD activity ( $p < 0.05$ ). Those in the highest tertile of plasma ferritin had also the lowest GPX activity but differences were not statistically significant. Plasma ferritin was significantly directly associated with plasma MDA levels and inversely associated with CuZn-SOD activity. Plasma ferritin levels was positively correlated with MDA levels and inversely correlated with CuZn-SOD activity. No associations were found between the tertile of plasma ferritin in GPX activity.

**CONCLUSION:** Our findings revealed an association between body iron stores and oxidative stress markers linked to atherosclerosis process. The results are also in agreement with the concept that iron overload would elevate the risk of coronary artery disease by promoting the lipid peroxidation.

**Keywords:** iron stores, ferritin, oxidative stress, atherosclerosis, women.

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## Introduction

In the scientific and public health communities, interest in iron overload subject has increased. Meanwhile, the proportion of iron-replete individuals in industrialized countries has been also raised.<sup>1</sup> The free iron might be harmful to cells because it catalyzes the generation of hydroxyl radicals ( $\cdot\text{OH}$ ) from superoxide ( $\text{O}_2^{\cdot-}$ ) and hydrogen peroxide ( $\text{H}_2\text{O}_2$ ).<sup>2</sup> The potential toxicity of iron derives from

its ability to serve as a catalyst in oxidation-reduction reactions, and its toxicity is enhanced by the limited capacity of the human body to excrete iron.<sup>3</sup>

There are several risk factors whose association with atherosclerosis is well established, including age, gender, dyslipidemia, smoking, hypertension, diabetes mellitus, obesity, and sedentary lifestyle.<sup>4, 5</sup> Yet, about 25 percent of CVD deaths in men and 15 percent in women occur in persons with multi-

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variate Framingham Study risk factors.<sup>6</sup> It has been suggested that about half of atherosclerosis cases cannot be attributed to standard risk factors.<sup>4</sup> This fact has recently stimulated a call for investigation of new risk factors.<sup>7,8</sup> In the last decade evidence has accumulated that a crucial and causative role in the pathogenesis of atherosclerosis is played by the free radical process known as lipid peroxidation. It is suggested that lipid peroxidation is involved in the oxidative modification of low density lipoprotein and this ultimately results in the formation of atherosclerotic lesions.<sup>9-10</sup>

It has been suggested that the risk of coronary heart disease increases with the increase of body iron stores. In support of this hypothesis, a prospective epidemiologic study of heart disease in Finnish men found that the risk of heart attack increased with the increase of serum ferritin level<sup>11</sup>. However, other studies have failed to support the hypothesis that high body iron stores would increase the risk of coronary heart disease.<sup>12, 13</sup>

Serum ferritin levels reflect the iron stores in the body. Free radical formation and lipid peroxidation can be prevented by the iron-chelating agents like desferrioxamine.<sup>14</sup> Cellular defense mechanisms against superoxides include series of linked enzyme reactions which remove the toxic radicals and repair radical-induced damage. The first enzyme is copper zinc superoxide dismutase (Cu Zn-SOD) which converts the superoxide anion into hydrogen peroxide. Hydrogen peroxide, that is toxic for cells, is removed by catalase (CAT).<sup>15</sup> A convenient and sensitive method for estimation of lipid peroxide concentration is the quantitative estimation of their metabolic end-product malondialdehyde (MDA) using the thiobarbituric acid method. MDA is one of the most frequently used indicators of lipid peroxidation.<sup>16</sup>

The purpose of this study was to investigate whether body iron stores has any relationship with the levels of oxidative stress markers. To test this hypothesis, we investigated body iron stores by measuring the concentrations of plasma ferritin. Furthermore, we assessed oxidative stress markers by measuring the concentrations of plasma MDA and activities of erythrocyte cytoprotective enzymes, including, Cu Zn-SOD, CAT and glutathione peroxidase, in a random sample of women in reproductive age without any cardiovascular disease.

## Materials and Methods

The samples were recruited from healthy women receiving the services of rural health centers of Kerman Province, Iran. One hundred sixty women aged 20-45 years (mean age 31.5 years) were randomly selected. The exclusion criteria was as following; pregnant and lactating women and those with history of cancer, cardiovascular disease, diabetes, renal or liver diseases, and those taking vitamin or mineral supplements. Informed written consent was obtained from subjects before entering the study. Demographic data were gathered using checklists and face-to-face interviews.

Body weight was measured to the nearest 0.1Kg while subjects were wearing neither shoes nor clothes. Body height was also measured to the nearest 1cm while subjects were not wearing shoes, in standing position. Body mass index (BMI) was calculated as weight (in kilogram) divided by height (in meters-squared). To calculate waist-to-hip ratio (WHR), the waist circumference was measured in a horizontal plane at the level of the high point of iliac crest to the nearest 0.1 cm. Hip circumference was measured in a horizontal plane at the maximum extension of the buttocks.

Venous blood samples were collected from the median cubital vein in standard tubes containing ethylene diamine tetra acetic acid (EDTA). Blood samples were centrifuged at 3000 rpm for 10 minutes at 4°C and plasma was separated for MDA assay. The buffy coat was removed and the remaining erythrocytes were washed three times in cold saline (9.0 g/l NaCl) and hemolyzed by the addition of cold deionized water. The subjects' plasma and hemolysate samples were stored at -70°C until analysis.

Plasma MDA concentrations were assayed by measurement of thiobarbituric acid reactive substances (TBARS) according to Satoh method.<sup>17</sup> The pink chromogen produced by the reaction of thiobarbituric acid with MDA was measured at 530nm. In order to express the activities of enzymes per gram hemoglobin (Hb), Hb concentration was measured in the hemolysates with a standard kit involving the cyanmethemoglobin method (Drabkin's method).

Catalase (CAT, E.C.1.11.1.6) activity was determined according to Hygo Aebi method.<sup>18</sup> Activity

of CAT was determined by following the decomposition of H<sub>2</sub>O<sub>2</sub> in phosphate buffer pH 7.2 by spectrophotometer at 230nm.

Glutathione peroxidase (GPX, E.C.1.11.1.9) activity was measured according to Paglia and Valentine method<sup>19</sup> and superoxide dismutase (SOD, E.C.1.15.1.1) activity was assayed using RAN-SOD kit (cat.NO.SD 125) (reference). Plasma ferritin concentrations were determined with Radioimmunoassay method and standard kit with the Ciba Corning ACS-180 analyzer.

In the present study, the 25<sup>th</sup> and 75<sup>th</sup> percentile values of plasma ferritin were used as threshold values to define 3 categories of iron stores; low (<25<sup>th</sup> percentile), medium (25<sup>th</sup> to 75<sup>th</sup> percentile), and high (>75<sup>th</sup> percentile).

The oxidative stress markers of interest were analyzed as continuous variables. Goodness of fit to normal distribution was investigated by probit plots and the Kolmogorov test. Comparisons of mean values of oxidative stress markers among the groups of iron stores were performed by the calculation of one-way analysis of variance (ANOVA). Pearson's correlation coefficient was applied to test relationships between plasma ferritin and oxidative stress markers. Because the oxidative stress markers of interest represent a cluster of indicators that collectively predict risk and may be correlated, we used multivariate analysis of variance in which the entire set of oxidative stress markers was considered as the dependent variable. A single *P* value was used to interpret the significance of the association between the set of oxidative stress markers and plasma ferritin concentration. This approach may be better than the consideration of each oxidative stress marker separately, because it adjusts for multiple comparisons and accounts for co-linearity between the dependent variables. As two comparisons were done (high versus medium and low versus medium plasma ferritin), a Bonferroni correction was applied. *P* values less than 0.05 were considered as statistically significant. SPSS software version 12.5 (Statistical Package for Social Sciences, SPSS Inc., Chicago, IL, USA) was used for all statistical analysis.

The statistical analysis part is too long; you can summarize it as following; Comparison of mean values of oxidative stress markers among the groups of iron stores were performed by one-way

ANOVA. Pearson's correlation coefficient was applied to test relationships between plasma ferritin and oxidative stress markers. *P* values less than 0.05 were considered as statistically significant. Statistical analyses were carried out using a statistical software package (SPSS; 12.5, Chicago, IL, USA).

## Results

The 25<sup>th</sup> and 75<sup>th</sup> percentile values for plasma ferritin (which were used to define low, medium, and high iron stores) were 23 and 68 µg/L for participants.

Mean values of oxidative stress markers according to tertiles of plasma ferritin concentration are shown in Table 1. Subjects in the highest tertile of plasma ferritin showed the highest levels of plasma MDA compared with subjects in the lower tertiles (*P*<0.05). Those in the highest tertile of plasma ferritin had also the highest mean values of CAT activity (*P*<0.05). Furthermore, Subjects in the highest tertile of plasma ferritin presented the lowest levels of CuZn-SOD activity (*P*<0.05). Those in the highest tertile of plasma ferritin had also the lowest GPX activity but differences were not statistically significant.

Plasma ferritin was significantly associated with plasma MDA levels ( $r=0.39$ , *P*<0.05) and inversely associated with CuZn-SOD activity ( $r=-0.25$ , *P*<0.05). Figure 1 illustrates the mentioned relationships. Adjusted mean values for the selected oxidative stress markers comparing women in the lowest and highest tertiles of plasma ferritin to those in the middle tertile are shown in Table 2 for the 2 models. In the first model, we matched subjects considering age. In the second model, we also matched for BMI, WHR and number of pregnancies. We observed that the aforementioned relationships remained significant even after adjustment for confoundings. Plasma MDA levels was significantly higher in the highest tertile of plasma ferritin compared with the middle tertile (*P*<0.05) with the Bonferroni adjustment for multiple comparisons. Furthermore, CuZn-SOD activity was significantly lower in the highest tertile of plasma ferritin compared with the middle tertile (*P*<0.05) with the Bonferroni adjustment for multiple comparisons. No associations were found between the tertile of plasma ferritin in GPX activity.

**Table 1:** Mean values of oxidative stress markers according to tertiles of plasma ferritin concentration.

Oxidative stress markers	Plasma ferritin tertiles (µg/L)		
	Low (<23 ) (n=62)	Medium (23-68 ) (n=56)	High (>68 ) (n=42)
MDA (µmol/L)	1.13±0.14	2.39±0.92*	3.47±1.02*
CuZn-SOD (U/g Hb)	836±68	673±56	587±71 #
GPX (U/g Hb)	98.1±3.3	93.2±4.1	89.7±3.6
CAT (K/g Hb)	165±54	198±47	219±58†

Values are mean±SD

\* p<0.001 compared to the lowest tertiles of plasma ferritin.

# p<0.01 compared to the lowest tertiles of plasma ferritin.

† p<0.05 compared to the lowest tertiles of plasma ferritin.

**Table 2:** Comparison of adjusted estimates (±SE) of selected oxidative stress markers by tertiles of plasma ferritin among women of reproductive age (20-45y).

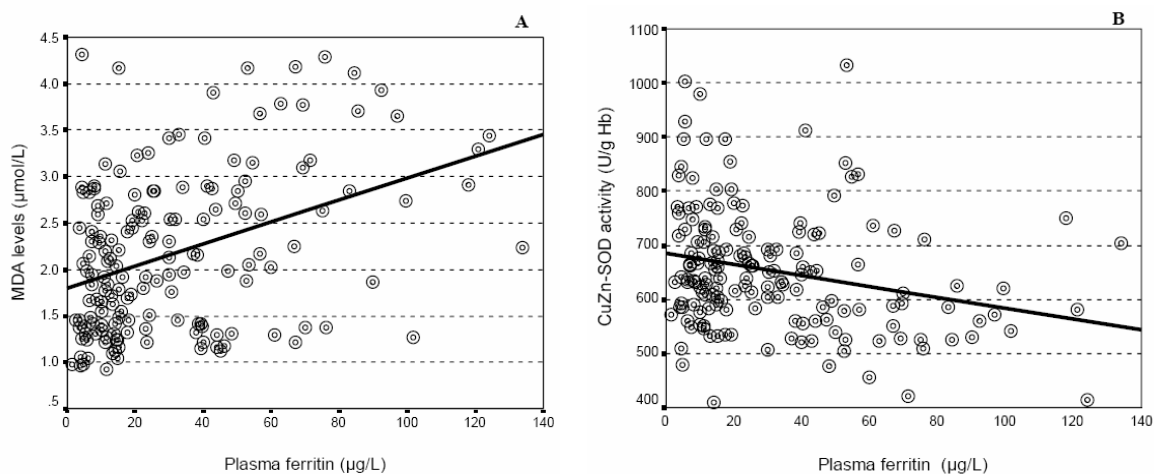
Plasma ferritin tertiles (µg/L)	MDA (µmol/L)	CuZn-SOD (U/g Hb)	GPX (U/g Hb)	CAT (K/g Hb)
<b>Model 1*</b>				
Low (<23 )#	-2.264±0.52	0.632±0.028	0.021±0.06	-1.652±0.61
Medium (23-68 )†	—	—	—	—
High (>68 )#	1.05±0.37	-0.841±0.044	-0.038±0.02	0.94±0.07
<b>Model 2**</b>				
Low (<23 )#	-1.167±0.032	0.325±0.025	0.041±0.03	-0.984±0.021
Medium (23-68 )†	—	—	—	—
High (>68 )#	1.149±0.481	-0.576±0.001	0.022±0.01	1.357±0.742

† Reference category

\* Adjusted for age

\*\* Adjusted for age, body mass index (BMI) and waist-to-hip ratio (WHR) and number of pregnancies

# p<0.01 (Bonferroni adjusted value for multiple comparisons) compared with medium plasma ferritin by multivariate analysis of variance in which the entire set of oxidative stress markers was considered as dependent variable.



**Figure 1:** Correlation between plasma ferritin and plasma MDA levels ( $r=0.39$ ,  $p<0.0001$ ) (A) and CuZn-SOD activity ( $r=-0.25$ ,  $p<0.001$ ) (B).

## Discussion

In this study we measured plasma levels of ferritin which reflects the quantity of storage iron of body, and MDA, a stable product of lipid peroxidation as an indicator of free radical generation in the human body. We investigated the relationship between ferritin and MDA.

The generation of free radicals is dependent on the presence of various transition metal ions.<sup>14</sup> The most important transition metals *in vivo* are believed to be iron and copper. To support the possible role of iron in the generation of free radicals *in vivo*, it has been shown that coronary reperfusion damage, a process thought to be partially mediated by reactive oxygen species, has been shown to be increased by iron load, and this damage was partially reversed by iron-chelating agents in experimental animal models.<sup>20</sup>

The most plausible explanation for an effect of iron is the stimulating of oxidation of low density lipoproteins (LDL).<sup>14</sup> During the last few years, oxidative modification of LDL has come into focus as an important step in rendering pathogenicity to LDL in atherosclerotic lesions.<sup>10</sup> There is considerable experimental evidence indicating that this modification increases the atherogenic effects of the LDL molecule. Several epidemiological studies have shown dietary antioxidants (especially vitamin E) to be inversely correlated with the incidence of coronary artery disease.<sup>21-22</sup>

Studies investigating whether iron status can be considered as a cardiovascular risk factor showed conflicting results, as reviewed recently<sup>23-24</sup>. This was not unexpected because none of the indicators of body iron status—hemoglobin, hematocrit, serum iron, transferrin, transferrin saturation, total iron binding capacity—accurately reflects body iron<sup>25</sup>. Because plasma ferritin concentrations are directly proportional to intracellular ferritin concentrations, it is considered to be the best clinical measure of body iron stores<sup>25</sup> and the most feasible one to use in epidemiologic studies<sup>26</sup>. However, few studies used plasma ferritin concentrations to examine whether body iron stores are associated with cardiovascular diseases. Plasma ferritin concentrations are known to be increased in response to inflammation. To circumvent a confounding effect of inflammation on Plasma ferritin concentrations, we

excluded subjects who had inflammation or other diseases.

The recently published study on the relation between serum ferritin and the risk of myocardial infarction suggests that iron might be important as a pro-oxidant in atherogenesis.<sup>11</sup> In our study; elevated Plasma ferritin concentrations were also associated with increase of oxidative stress. Similarly, Yesilbursa et al found positive relation between Plasma ferritin and MDA concentrations in patients with CAD.<sup>27</sup> Extensive reviews have been recently published concerning the role of iron in free radical reactions, such as lipid peroxidation<sup>14, 28</sup>. We have also shown that Subjects in the highest tertile of plasma iron presented the highest levels of plasma MDA.<sup>29</sup> Iron in body largely stored in ferritin. In essence, ferritin acts as a critical antioxidant defense by sequestering unbound or "free" iron, thereby limiting its participation in damaging oxidative reactions. Iron loading at the cell level induces a 10-fold increase in intracellular ferritin<sup>30</sup>. The induction of ferritin in endothelial cells is cytoprotective against oxidative injury by sequestering and inactivating of iron. To promote free radical production, iron must be liberated from ferritin, but body iron is so tightly bound that there may not be free iron available *in vivo* under physiological conditions. It is believed that oxidant stress itself can provide the iron necessary for formation of reactive oxygen species, for example, by mobilizing iron from ferritin.<sup>27</sup> For instance, Superoxide radicals ( $O_2^-$ ) have been observed to liberate iron from ferritin by reducing ferritin-bound  $Fe^{+3}$  to  $Fe^{+2}$ , whereupon it is released from ferritin and becomes available to catalyze self-propagating burst of oxidation.<sup>31</sup> The  $O_2^-$  derived from stimulated granulocytes or generated by xanthine oxidase is able, in this way, to mobilize iron from ferritin.<sup>32</sup> Iron can also be released from ferritin at low extracellular PH or by arterial wall damage.<sup>33-34</sup>

(when you mentioned a part of result in your discussion, you should mention other studies that are agreed or not agreed with your study not the pathophysiology of the result, it is better to omit the highlighted part and use other studies).

We then investigated the relationship of body iron stores with the activities of erythrocyte antioxidant enzymes. We observed that subjects in the highest tertile of plasma ferritin levels had the low-

est level of CuZn-SOD activity and highest level of CAT activity. To our knowledge, this is the first study to investigate the relation between plasma ferritin levels with the activities of erythrocyte antioxidant enzymes in an apparently healthy human population, and therefore, we could not compare our data with other epidemiological studies. Nevertheless, we did not observe any relation between the changes of plasma iron and the GPX activity. However, some researchers observed increased level of CuZn-SOD activity in patients with iron deficiency anemia who had lower iron stores<sup>35-36</sup>. It is well known that reactive oxygen species, especially hydrogen peroxide, inhibit SOD activity.<sup>37</sup> Furthermore, decreased SOD activity may contribute to free radical production. CAT is an iron-dependent enzyme and would be expected to be increased with elevation of body iron stores.

Although the cross-sectional design of this study precludes inferences of causality, it is not likely that the observed associations were confounded by all factors of which were controlled for the analysis. The choice of plasma ferritin as a valid indicator of iron stores is a potential concern. Finally, another strength of the study design is that the study sample consisted of relatively healthy women of reproductive age.

Our study may have some limitations in data gathering like all cross-sectional studies. First, as with all observational studies, our results could be biased by unrecognized confounders. Second, we couldn't assess nutrient intakes (including antioxidant nutrients) of participants.

In conclusion, we observed plasma ferritin concentrations to be associated with increased oxidative stress. The results are also in agreement with the concept that iron overload would elevate the risk of coronary artery disease by promoting the lipid peroxidation. Prospective studies, especially intervention studies that ensure adequate iron status, are needed to compare 2 possible scenarios; 1) improved iron status increases the risk of CVD, or 2) women with a higher risk of CVD have higher iron stores, with both factors resulting from the same underlying cause.

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