Abstract: Background- Iron is a trace element essential for the body because it is an important constituent of haemoglobin which is a carrier of oxygen in the body. Iron has also been found to cause harmful effects when it is found in excess since it increases the production of free radicals which produce oxidized LDL and lipid peroxides involved in the pathogenesis of atherosclerosis, which is a chronic inflammatory disease. Peripheral vascular disease (PVD) is a prototype of chronic systemic atherosclerosis characterized by stenoses and occlusions in the peripheral arterial bed of lower limbs. A number of researches have been undertaken to determine the association between the status of body iron and coronary atherosclerosis. But there is little consensus about the causal relationship in the studies conducted so far. The objective of this study was to find out the Iron status in atherosclerotic peripheral vascular disease by measuring serum ferritin in patients attending our hospital. This could serve as a representative of the south Indian population. Methods- This case-control study was conducted in the department of Vascular surgery in Rajiv Gandhi Government General Hospital, Chennai. 50 patients of atherosclerotic peripheral vascular disease, defined by Ankle Brachial Pressure Index 0.9 were included in the study. The controls were 50 healthy subjects matched for age and sex recruited. Serum Ferritin was measured using Immunoturbidimetry. The reference values are 20-250 g per L for men and 20-200 g per L for women. Results- The mean serum ferritin level in cases and controls were 203.3 g per L and 180.5 g per L, respectively. Student t test was used to calculate statistical significance and no significant difference was found in ferritin levels between cases and controls. Conclusion- Serum ferritin is not a significant risk factor for atherosclerotic peripheral vascular disease in the study population. 

Keyword: Iron status, athrosclerosis, ferritin, free radicals, inflammation

Background: Atherosclerosis is a chronic inflammatory disease. Cardiovascular atherogenicity is the major cause of mortality around the world, though it can affect all the medium and large sized vessels in the body. Dyslipidemia is a major risk factor for the development and progression of atherosclerosis, along with other risk factors like age, male sex, smoking, alcoholism, sedentary life style, dietary habits and co morbid conditions like Diabetes mellitus and hypertension. The relationship between iron status and atherosclerosis has been a topic of debate in the literature. Majority of studies have focused on iron burden with respect to a role in the onset and/or progression of coronary artery disease (CAD). The effect of iron in the coronary arterial system may, however, differ from its effect in the peripheral arterial system.

Peripheral Vascular disease, commonly referred to as Peripheral Arterial Disease (PAD) or Peripheral Artery Occlusive Disease (PAOD), refers to the obstruction of large arteries not
within the coronary, aortic arch vasculature, or brain. PVD can result from atherosclerosis, inflammatory processes leading to stenosis, an embolism, or thrombus formation. The prevalence of atherosclerotic PVD has been on the rise and especially in the aged and diabetics. It is becoming a major public health issue in developing countries like India. The prevalence of PVD in south India is 3.2% in general population and 7.8% among known diabetic patients (1).

Iron is a trace element with an atomic number of 26, atomic weight of 55.85 and is said to be invariably required by every human cell. It is quantitatively an important biocatalyst element with vital roles in oxidative metabolism, cellular growth and proliferation and oxygen transport (2). Careful homeostasis of iron metabolism is critical as both iron-deficient (eg.anemia) and overload (e.g. hemochromatosis) states can be deleterious to the human body. 66% of body iron is incorporated in hemoglobin in the ferrous ($Fe^{+2}$) form. 27% of the body’s iron is incorporated as tissue ferritin in the ferric ($Fe^{+3}$) state (3). When the intracellular storage capacity is exceeded, additional intracellular iron is incorporated into hemosiderin and, to a degree, iron is sequestered in the reticuloendothelial system (4). Iron in the circulation is bound to transferrin, a protein that delivers iron to cells. Free ferrous ($Fe^{+2}$) iron may catalyze a variety of free-radical oxidative reactions, which in turn may promote a variety of degenerative processes (5-7). Ferrous iron may participate in a set of reactions known as the Fenton or Haber–Weiss reaction and catalyze the net formation of hydroxyl radicals (OH·) from superoxide (O2·-) and hydrogen peroxide(H2O2) (6,8,9). During times of oxidative stress these reactive oxygen species (ROS) can liberate iron from its stable forms of ferritin and heme, respectively (4,9). Free iron may also be more available in acidic, low pH environments where its affinity for transferrin is reduced (10).

The common indicators of body iron status are serum Iron, Total Iron Binding Capacity (TIBC) and serum Ferritin. Of these, serum Iron and Transferrin are not very reliable indices because their level can change grossly over a short period of time even in healthy people due to momentary imbalances in iron inflow and outflow. There is a diurnal and significant day to day variation in iron levels and it is also affected by a wide range of clinical conditions (2). The measurement of serum ferritin provides the most useful indirect estimate of body iron stores (2, 18). In normal circumstances the amount of plasma ferritin seems to be proportional to the

$$O_2 + Fe^{+2} \rightarrow O_2^{+3} + Fe^{+2}$$

Fenton reaction

$$Fe^{+2} + H_2O_2 \rightarrow Fe^{+3} + OH^- + OH$$

Haber–Weiss reaction

$$O_2 + H_2O_2 \rightarrow O_2 + OH^- + OH$$

Hydroxyl radicals have been implicated in low-density lipoprotein (LDL) cholesterol oxidation (6,11). Circulating tissue macrophages readily internalize oxidized LDL via scavenger receptors and evolve into foam cells, the key components of the early atherosclerotic subendothelial plaque (12,13). In addition, oxidized LDL attracts circulating monocytes to the arterial endothelium and exerts a static force on macrophages to prevent their migration from the evolving plaque in the vessel wall (14). Reif and Simmons have shown that in the presence of sodium nitroprusside (a NO precursor), iron is released from ferritin. This increased availability of free ferrous iron may thus increase the potential for iron-catalyzed oxidative reactions (15). Ambrosio and others have suggested that the ROS generated by the Fenton or Haber–Weiss reactions may contribute directly to plaque disruption and thrombosis (16). Pratico et al demonstrated that Fe$^{+2}$ could induce platelet aggregation (as well as thromboxane B2 and protein kinase C) in a dose dependent manner (17).
amount of cellular ferritin and under most circumstances accurately reflect the amount of storage iron present (19). Ferritin is also an acute phase reactant and its level is found to be elevated in fever, acute and chronic infections and liver diseases (2,19).

Several studies in the past have evaluated the relationship between iron status with coronary artery disease with equivocal results. The following are a few examples. Haidari et al found that ferritin was an independent discriminating risk factor for CAD (20). Say et al found that hypercholesterolemic subjects with, as opposed to those without significant stenosis had significantly higher ferritin levels (21). Salonen found that high ferritin levels, even when adjusted for markers of inflammation, were directly associated with risk of myocardial infarction (22,23). In the Helsinki Heart Study Manitari found that serum ferritin showed no association with non-fatal myocardial infarction or cardiovascular death (24). In a preliminary prospective analysis of 238 US physicians who developed myocardial infarction over a follow-up of 8 years, ferritin levels did not differ significantly at baseline compared with age-matched controls (25). Hence this study was undertaken to observe the association of serum ferritin with atherosclerotic peripheral vascular disease in this south Indian population.

Materials and Methods:

This case-control study was conducted in our Hospital from August to September 2011. 50 patients (47 male, 3 female, mean age 52 ± 10 yrs) of atherosclerotic peripheral vascular disease were included in the study. The patients had symptomatic PVD and were diagnosed using Ankle Brachial Pressure Index <0.9, and were further subjected to radiological examination of the affected vessel. Patients with acute and chronic infections, inflammatory disorders, liver and renal diseases, malignancies, hemoglobin <12 g/dL, those on hematinsics, were excluded from the study. 50 healthy subjects (Controls) matched for age and sex were recruited. Detailed history was taken from all the subjects, their height and weight recorded and they were subjected to a general clinical examination. 5 mL of blood was collected into plain vacutainer tubes by venipuncture under aseptic conditions. Blood was allowed to clot and serum was separated after centrifugation. A part of this unhemolyzed serum was stored in Eppendorf tubes and kept frozen at -20°C for estimation of ferritin. The remaining serum was used for estimation of the various lipid parameters Total cholesterol, Triglycerides, High Density Lipoprotein (HDL) and LDL, within 6 hours of blood collection.

Total Cholesterol, Triglyceride and HDL were estimated using appropriate kit methods in a semiautoanalyzer Erba Chem 7.

LDL was calculated using the Friedwald’s formula which is LDL in mg/dL= Total Cholesterol- (Triglyceride/5 + HDL) Serum Ferritin was estimated using Immunoturbidimetry. Prior to analysis the samples were brought to room temperature and thawed. The principle of this analysis is agglutination of latex particles coated with anti-human ferritin antibodies by the ferritin present in the serum sample. This agglutination would be proportional to the concentration of ferritin in the sample and the absorbance was measured at 540 nm using a spectrophotometer. The reference range is 20-250 g/L for men and 20-200 g/L for women.

Results

Statistical analysis was performed using the SPSS software for PC. The mean, standard deviation and other statistical parameters were calculated and student’s ‘t’ test was used to calculate the statistical significance in the values of ferritin between cases and controls. P value of <0.05 was considered significant. Pearson’s correlation test was done to see the association of ferritin levels with the lipid parameters.
The mean level of serum ferritin (g/L) in cases of PVD was 203.3 ± 72.8 and in controls 180.5 ± 46.3. This difference was not statistically significant, though the mean ferritin level is higher in cases than controls. Statistically significant difference was observed in the conventional risk factors like Body Mass Index, Total Cholesterol, Triglyceride, HDL and LDL cholesterol. HDL was lower in the cases while all the other parameters significantly higher in the control population. The results are shown in table 1.

Table 1. Comparison of ferritin and other risk factors between PVD and normal subjects

<table>
<thead>
<tr>
<th>PARAMETER</th>
<th>CASES (n=50)</th>
<th>CONTROLS (n=50)</th>
<th>p VALUE (&lt;0.05 sig)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ferritin (g/L)</td>
<td>203.3 ± 72.8</td>
<td>180.5 ± 46.3</td>
<td>0.085</td>
</tr>
<tr>
<td>BMI</td>
<td>25.7 ± 4</td>
<td>23.4 ± 2</td>
<td>0.001</td>
</tr>
<tr>
<td>T Cholesterol (mg/dL)</td>
<td>195.1 ± 47.3</td>
<td>197.5 ± 17.7</td>
<td>0.001</td>
</tr>
<tr>
<td>Triglyceride (mg/dL)</td>
<td>190 ± 47.7</td>
<td>113.2 ± 18</td>
<td>0.001</td>
</tr>
<tr>
<td>HDL</td>
<td>119.7 ± 44.3</td>
<td>80 ± 18.4</td>
<td>0.001</td>
</tr>
</tbody>
</table>

* no significance

There was no correlation between the ferritin levels and any of the above parameters. It can be seen clearly from the above table that elevated Body Mass Index (weight in kg/ height in m²), elevated serum cholesterol, triglycerides and low density lipoprotein cholesterol and decreased high density lipoprotein cholesterol are highly significant as risk factors for PVD. The levels of ferritin and lipid profile parameters are illustrated in Figure 1.

Fig 1. Ferritin and Lipid parameters in cases and controls

With respect to history, smoking and alcoholism were more prevalent in cases than controls, 80% of cases and 28% of controls were smokers and 86% of cases and 30% of controls were alcoholics.

Discussion

This study has re-emphasized the association of established risk factors for atherosclerosis, like male sex, smoking, alcoholism, obesity, and dyslipidemia. Though there is no statistically significant difference in ferritin levels, cases have a mean value higher than that of controls, not excluding the possibility of a causal relationship between iron stores and atherosclerosis in PVD.

The lack of significant association of ferritin with atherosclerotic PVD is supported by a number of earlier studies. A cross sectional study and case control study in Atherosclerosis Risk in Communities (ARIC) study found no association between ferritin and carotid artery atherosclerosis (26). A meta analysis of 12 prospective studies found no evidence to support an association between biomarkers of iron metabolism and coronary heart disease (27). Recently, a multicenter randomized controlled trial was conducted with 1,277 men and postmenopausal women randomized to either a control group or to receive phlebotomy at six-month intervals to reduce body iron stores; at the end of the six year followup, all-cause mortality and the secondary endpoint of all cause mortality plus nonfatal myocardial infarction and stroke were nonsignificantly lower in the phlebotomy group (28).

Most of the subjects in both cases and controls had ferritin within the normal range, only 12% of cases and 10% of controls had values in excess of normal. The present study has got its limitations. The study population is a small subset of south Indian population, where Iron deficiency is very common due to malnutrition and occult intestinal bleeding due to worm infestation and Aspirin therapy etc. This warrants the need for a large scale study covering various ethnic groups from various parts of India.
To determine the associative or causal role of iron in atherosclerosis, a well designed, randomized controlled trial with complete measurements of serum iron, TIBC, ferritin, and transferrin receptor should form the basis of the investigation. Recently, measurement of the soluble fragment of transferrin receptor has gained popularity as a more valid assessment of iron deficiency. Not only have increased levels of this receptor been noted in the cell’s initial response to iron-deficient states but also it is not influenced by chronic inflammation or infection (29,30). The serum transferrin receptor:serum ferritin (TfR/ferritin) ratio, may be the most reliable indicator of iron status in iron-deficient, iron-replete, and iron-overloaded states (30).

Conclusion
This study shows there is no significant association of serum ferritin with atherosclerotic peripheral vascular disease. Iron may have an associative role in the genesis and growth of the atherosclerotic plaque, but its effects in the final stages of vascular occlusive disease are less evident. Further investigation into the role of serum iron in intermittent arterial claudication may provide useful insight. Clinical primary or secondary endpoints in future studies may also include interim measurements of the peripheral artery vessel walls via high-resolution B-mode ultrasound imaging. Primary and secondary prevention studies focusing on PVD and iron status with clinically meaningful outcomes are needed.

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