IRON HYPOTHESIS OF CARDIOVASCULAR DISEASE:
STILL CONTROVERSIAL

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(Abstract): Iron hypothesis has been a controversial subject for over 30 years as many studies support its role as a risk factor for cardiovascular disease, while other studies found no evidence to support it. The conflicting results are accounted for by the non-homogeneity of trial design in terms of population inclusion criteria and different endpoints, non-uniform use of parameters for assessing iron role, and incomplete understanding of the mechanisms of action. The nature of iron is dual, being of crucial importance for the human body, but also toxic as "free iron" induces oxidative stress. Under physiological conditions, there are efficient and complex mechanisms against iron-induced oxidative stress, which could be reproduced for creating new, intelligent antioxidants. Iron depletion improves the cardiovascular prognosis only if serum concentration is at the lowest limit of normal ranges. However, low iron levels and the type of dietary iron intake correlate with atherosclerotic cardiovascular disease, influence the ischemic endpoints in the elderly, and exert negative impact on heart failure prognosis. So far, the causal relation and involved mechanisms are not fully elucidated. Iron overload is a difficult and frequent condition, involving the cardiovascular system by specific pathogenic pathways, therefore determining a particular form of restrictive cardiomyopathy and vaso-occlusive arterial damage. Keywords: IRON HYPOTHESIS, OXIDATIVE STRESS, CARDIOVASCULAR DISEASE, IRON DEFICIENCY, BODY IRON STORES

IMPORTANCE OF IRON
IN THE HUMAN BODY

Although the total amount of human body iron is very small (4-5 g), it is of vital importance. Iron is a metabolically active micronutrient with the unique property to donate and accept electrons, so it exists in a range of oxidation states, the most common being the bivalent or ferrous (Fe²⁺) and trivalent or ferric (Fe³⁺) forms. The human body uses this property in the main enzymatic and non-enzymatic cellular processes. Dietary iron is absorbed mainly by duodenal enterocytes using specific transmembrane transport mechanisms, depending on the heme or non-heme iron form. Systemic iron metabolism regulation is controlled by hepcidin through the specific receptor ferroportin which optimizes and synchronizes its prompt use, preserves iron stores, and prevents uptake by enterocytes. Over 80% of circulating iron is directed to hemoglobin synthesis, whereas the remaining 5-15% provides structure and function
to various enzymatic proteins (cytochromes, myoglobin, iron-sulfur complexes). Therefore, iron is involved in the proper oxygen transport and storage, regulation of cellular energy processes, cellular apoptosis and growth, inflammation, gene regulation, DNA and neurotransmitter synthesis, myogenesis, and xenobiotic metabolism. The action of iron is achieved by binding to transferrin, the specific "carrier" which delivers it to target cells using soluble receptors – sTfR. In the absence of a physiological pathway for excretion, iron excess is stored as ferritin (10-20%), mainly in the liver, spleen and bone marrow cells. Intracellular iron metabolism is regulated by a complex regulatory protein system (1, 2).

**IRON HYPOTHESIS**

The role of iron in cardiovascular disease was first mentioned in the ‘60s, when the relationship between iron deficiency anemia in inflammatory diseases and heart failure (HF) was postulated, but the issue was abandoned (3, 4). The American researcher and clinician Jerome L. Sullivan is the first to rediscover and theorize this relationship based on a series of clinical observations, namely that HF is usually a rule in iron overload diseases, and iron stores are higher in men and post-menopausal women. Sullivan gathered these observations in the “iron hypothesis” published in 1981. This hypothesis states that postmenopausal women are at higher risk for cardiovascular disease not only by changing their hormonal profile, but also due to the accumulation of stored iron with age (5).

Based on studies available at that time (6, 7), Sullivan proposes a new paradigm that iron may modulate the ischemic risk and atherogenesis through a unifying mechanism (8). The pathogenic mechanism of the paradigm is oxidative stress. Iron has the propensity of binding reactive oxygen species through Fenton reaction: $\text{Fe}^{2+} + \text{H}_2\text{O}_2 = \text{Fe}^{3+} + \text{OH}^- + \text{HO}^\cdot$ and Haber-Weiss reaction: $\text{O}_2^- + \text{Fe}^{3+} = \text{O}_2 + \text{Fe}^{2+}$. Hydroxyl radical (HO\(^\cdot\)) resulting from these reactions is most harmful to cells and tissues. However, the cytotoxic potential of iron is possible only in case of free iron, also known as "serum catalytic iron". Under physiological conditions, the human body neutralizes free iron by its complete binding to transferrin and ferritin. Any situation in which storage capacity is exceeded or there is an inadequate iron supply will result in overproduction of reactive oxygen species, with cytotoxic effect on cardiomyocyte and arterial wall. The relationship is bidirectional because vascular inflammation, common in cardiovascular disease, also induces oxidative stress. The consequence is the stimulation of iron release from ferritin and low affinity of transferrin for iron, namely generation of circulating catalytic iron. The mechanism of action of free iron on arterial vessel is the subject of numerous experimental studies. Some studies show that iron induces endothelial dysfunction, the promoter of atherosclerosis (ATS). In an original study, Kartikasari et al. noticed the increasing rate of monocyte adhesion to human umbilical endothelial cells treated with serum from patients with hemochromatosis, followed by the expression of adhesion molecules (VCAM, ICAM, E-selectin) in direct relation to the level of free iron (9). Recently, in a study investigating endothelial dysfunction and inflammation induced by iron oxide nanoparticles, Zhu et al. confirmed the toxic action of catalytic iron on human aortic endothelial cells. The authors suggested that the
interaction was mediated by monocytes that release free iron through phagocytosis, but the nanoparticles also have a direct structural and functional cytotoxic effect on vascular endothelium (10). An interesting observation reported by other studies on iron deficiency is the increase in nitric oxide production and iNOS synthase activity that paradoxically alter the intracellular metabolism and induce the release of catalytic iron (1, 10). The relationship with the initiation of atherogenesis is another process demonstrated in the 80’s and is based on LDL oxidation and formation of fatty streaks, as the earliest stage in atheroma plaque formation. Recent studies also support iron intervention in ATS progression. Exposure of red blood cells to oxidized plaque material induces erythrocyte lysis and generation of ferrohemoglobin, which is converted by oxidation to ferri- and ferril-hemoglobin. This chain of chemical reactions destabilizes the heme group that is oxidatively cleaved and releases iron. Thus, plaque lipid oxidation is a feed-forward process which promotes atheroma development, while free iron exerts its cytotoxicity on endothelial cells. Interestingly, the lipids of atheroma plaque isolated from the arterial vessel are not harmful to endothelial cell, while the exposure to red blood cells induces cytotoxicity (11).

LESSONS FROM CLINICAL TRIALS

Iron hypothesis is even today a topic of debate and controversy as there are numerous studies that equally support or deny the initial view. The first remarkable study that supports the iron hypothesis is KIHD, a Finnish prospective trial that investigated over 5 years a cohort of 1,931 men aged between 42 and 60 who had no symptomatic ischemic heart disease (IHD) at entry (12). The conclusion is that serum ferritin level $\geq 200 \, \mu g/L$ (considered to be normal) in subjects with high dietary intake of red meat is associated with a 2.2-fold higher risk of acute myocardial infarction (AMI), and the effect is enhanced by LDL-cholesterol. The findings of this reference study are also supported by the Rotterdam trial (13). It supports the relationship with the risk of AMI in the elderly, but again emphasizes the synergistic role of serum ferritin and cardiovascular risk factors, like diabetes, smoking, high LDL-cholesterol. As there are many conflicting data, two recent meta-analyses synthesized the information regarding the relationship between IHD and ferritin level (11,337 patients) or total iron-binding capacity with transferrin (14, 15). The conclusion is that each 50 $\mu g/l$ increase in ferritin above the level of 200 $\mu g/l$ increases by 2.4% the IHD risk, and the effect is synergistic with other cardiovascular risk factors (14). As to transferrin, considered an antioxidant molecule due to its ability to bind free iron, an inverse relationship between its level and IHD risk was found (15). The role of ferritin in promoting atherothrombosis is demonstrated by studies focused on the risk of carotid disease. SHIP study suggests a relationship with the prevalence of carotid damage in 2,443 participants recruited from the general population (16). The results are in agreement with the Bruneck study, the first of this kind, in which ferritin is considered to be one of the strongest predictors of incidence and progression of preexisting carotid lesions assessed by ultrasonography for 5 years, its effect being also amplified by LDL-cholesterol (17).

However, there are just as many studies suggesting that iron has a neutral effect on
cardiovascular risk (18). Meta-analyses and studies offer several explanations for these conflicting results (19). The first would be non-homogeneous study design related to different inclusion criteria, cutoffs used to define iron deficiency/overload, various end-points such as all-cause mortality, IHD, AMI mortality, stroke. A second key issue arises from the method to assess iron status (in most studies by measurement of ferritin, but also serum iron, transferrin saturation, dietary iron intake etc) and the interpretation of results (related to the impact of other risk factors). A good example is the study conducted by Sung et al. showing that ferritin is independently correlated with early coronary ATS estimated by calcium score, when not only the influence of traditional risk factors but also transferrin, markers of inflammation, and other risk factors are eliminated (alcohol, diet, type of physical activity, components of the metabolic syndrome) (20). The limits of interpretation might be related to the markers used to define iron role. For example, currently there are only two epidemiological studies using the sTfR/ferritin ratio, considered optimal for iron status description in the human body. These studies noted the weak but consistently positive relationship with the risk of carotid ATS or the occurrence of a first AMI (21, 22). A promising perspective is the use of catalytic iron as a marker of cardiovascular risk, based on observations of two recent studies (23, 24). Rajapurkar et al. support both the relationship between catalytic iron levels and the prevalence of cardiovascular disease and its importance in acute coronary syndromes (25). The 10-month follow-up of a cohort of 1,701 patients with acute coronary syndromes concluded that the new marker is able to detect early a heart attack (within 3 hours of the onset of chest pain) and to identify the high-risk subgroup for all-cause mortality (3.97-fold) and adverse cardiac events apart from traditional risk factors. Finally, the results could be influenced by information related to the mechanisms of action of iron, insufficiently used in studies. For example, there are voices that argue that iron does not increase the cardiovascular risk directly but by amplifying the harmful vascular effects of homocysteine, assessed by flow-mediated vasodilatation (26). In an article published this year, Hsieh et al. report remarkable findings regarding hepcidin, which acts as an intrinsic cardiac hormone with a predominantly local effect. The authors note increased hepcidin levels in human cardiomyocytes pretreated with ferrous iron, which has a cytotoxic effect. The conclusion elegantly demonstrates that hepcidin can protect cardiomyocytes from iron-induced apoptosis through the extrinsic apoptotic pathway and GATA-4/Bcl-2 inactivation, a myocardial survival factor (27). Therefore, hepcidin contributes to the maintenance of myocardial structure. This study completes and offers a molecular explanation for previous data regarding the low level of hepcidin and increased 3-year mortality in HF or its correlation with increased left ventricular mass (28). On the other hand, there are observations on up-regulation of hepcidin specific to ischemic myocardium after myocardial infarction (29). In the light of the study by Hsieh, it can be speculated that increased levels of hepcidin have the potential to diminish catalytic iron cytotoxicity derived from oxidative stress, thus limiting the infarct size (27). Finally, an interesting conclusion is that chemotherapeutic agents such as daunorubicin and doxorubicin induce cardiomyopathy by the same mechanism of iron-induced GATA-4 inactivation, namely feroptosis.
SERUM IRON LEVEL – PROTECTOR OR RISK FACTOR?

Based on the results of studies supporting the role of ferritin as a cardiovascular risk factor at levels close to the upper limit of the normal range, Sullivan launches a new assertion on the possible protective role of iron depletion against ischemic events, ATS lesions included (30). Salonen supports the idea reporting the reduction of the risk of ischemic events in all arterial areas, and of the number of revascularization procedures (12), and Meyers demonstrates the reduction of the risk of fatal AMI in men blood donors versus non-donors (31). Latest data from the multicenter controlled randomized trial conducted at the Department of Veterans Affairs Cooperative Studies Program on 1,277 patients with stable symptomatic peripheral arterial disease also suggest that reducing iron stores could improve the clinical evolution in certain subgroups of patients – young, smokers with low average concentrations of ferritin (32). Basically, iron depletion improves the cardiovascular prognosis only if serum concentration is at the lower limit of normal ranges.

Iron deficiency is well-documented to be associated with increased cardiovascular risk. Recent studies support the relationship with the high prevalence of IHD and carotid ATS, the influence on the ischemic endpoints in the elderly (33, 34), and its prognostic role in HF (35). The unsolved problem remains whether serum iron is causally related to cardiovascular disease, or is only a marker of chronic diseases associated with cardiovascular risk (19). Iron deficiency causes thrombocytosis associated with the risk of thrombotic complications (36), hypercoagulable state due to reduced deformability of microcytic erythrocytes, and increased viscosity (19). Finally, an important mechanism is iron deficiency anemia, a pejorative factor in the higher incidence and progression of chronic HF (2). Anemia in HF is the consequence of absolute iron deficiency resulting from decreased dietary intake, impaired intestinal absorption and transport mechanisms, blood loss after antiagregant and anticoagulant medication, abnormal hepcidin regulation etc. At the same time, HF is a state characterized by inflammatory status associated with increased levels of pro-inflammatory cytokines and oxidative stress. Inflammation leads to inadequate iron supply to meet the demand despite normal body stores, a condition called functional deficiency. Renal impairment and associated infections maintain inflammation and consequently iron deficiency. The fact is that anemia causes hypoxia, which induces increased nitric oxide concentrations causing vasodilatation and low blood pressure. It results in sympathetic stimulation that causes change in renal hemodynamics with decreased glomerular filtration rate, stimulation of renin-angiotensin-aldosterone system, and ADH secretion. The consequence is fluid retention and plasma volume expansion with a negative impact on the structure and function of the heart. Heart dilatation, increased ventricular mass, and apoptosis of cardiomyocytes are common events associated with cardiac dysfunction (35). Therefore, the association of anemia with HF and renal dysfunction (cardio-renal syndrome) is suggestively called the "triangle of death" (37). Despite this well-known pathogenic substrate, iron deficiency in HF was less studied. Opasich et al. noted that 20% of patients with systolic HF had absolute iron deficiency microcytic anemia, but in
57% of patients it was due to functional iron deficiency (38). Shortly after, Nanas published the only study to date in which iron deficiency was assessed by the standard method, bone marrow biopsy, and reported a high percentage (73%) in patients with advanced HF (39). Until now, only two observational studies reported the incidence of iron deficiency in the general population with HF, which seems to be more common in women, in advanced stages of disease with high NT-proBNP and C-reactive protein levels (40, 41). The fact remains that iron deficiency with or without anemia affects the myocardium and skeletal muscle, both organs having increased energy needs dependent on iron normal metabolism. Experimental data show that the myocardial molecular consequences are an increased expression of natriuretic peptides (ANP, BNP) and hepcidin, extracellular matrix remodeling, mitochondrial dysfunction, apoptosis, and reduced expression of TfR-1 (2,42). In the skeletal muscle, iron deficiency causes low oxygen transport and diffusion, decreased oxidative capacity, abnormal use of energy sources represented by glucose and fatty acids (2). The clinical consequence is decreased exercise ability. Also interesting are the clinical data that support the correlation with the risk of depression, predominantly in males (41). Also important is the negative impact on prognosis, iron deficiency being a strong predictor of the need for transplantation and 3-year risk of all-cause mortality (41, 43). On the other hand, the findings of seven studies on iron supplementation benefit in these patients are promising (2). Although the number of patients included is small, the follow-up period is short, and study design is heterogeneous, there is the assumption that iron deficiency should become a therapeutic target in HF. Its correction improves exercise ability, NYHA class, systolic dysfunction assessed by echocardiography, and mortality. FAIR-HF trial, the only multi-center placebo-controlled study that included both patients with and without anemia followed for 24 weeks, supports the beneficial effect of i.v. iron supplementation regardless of NYHA class, HF etiology, co-morbidities, hemoglobin level, with important impact on rehospitalization rate and good clinical tolerance (44). Similarly, a meta-analysis by Hunnicutt et al. published this year suggests that the incidence of IHD is related to low iron concentration and dietary heme iron intake. The causal relationship and the mechanisms involved remain to be elucidated (45). Lapice believes that the relationship with morbidity and mortality from IHD is not directly related to iron, but to dietary and lifestyle behaviors (smoking, alcohol consumption, level of physical activity) that can modulate the risk. For example, red meat containing heme iron has high levels of saturated fats and other oxidants (19).

Iron overload is a much more difficult to handle both clinically and therapeutically and the cardiovascular involvement is demonstrated by such iron storage diseases as hemochromatosis. In practice, there are also frequent conditions which require repeated blood transfusions, causing iron accumulation (hemosiderosis) in the absence of physiologic iron excretion mechanism. A unit of blood is equivalent to a dietary intake of 200 mg iron. A total body iron of over 7 g, corresponding to 30-50 units of red cells, can lead to signs of tissue toxicity. For this reason, it is necessary to monitor serum ferritin levels and at > 1000 μg/l, iron phlebotomy and chelating therapy become man-
Iron hypothesis of cardiovascular disease: still controversial

datory (46). The cardiac response to iron overload is cardiomyopathy, typically developing in two stages - initially restrictive cardiomyopathy caused by fibrosis and myocardial ischemia invariably progressing to dilated cardiomyopathy with severe biventricular impairment (47). Diastolic dysfunction is considered an independent prognostic marker for the increased mortality rate in this category of patients. The pathophysiology of iron overload cardiomyopathy is mediated by oxidative stress that enables ischemia-reperfusion lesions even in the presence of normal coronary arteries. Specifically, iron regulates excitation-contraction coupling in cardiomyocytes via action on L-type Ca\(^{2+}\) channels, thereby causing diastolic and systolic dysfunction (48). Associated chronic tissue iron deposition and its electrophysiological effects determine interstitial fibrosis and cardiac specific conduction system damage, the cause of arrhythmias with prognostic impact. On the other hand, oxidative stress causes vascular impairment secondary to endothelial dysfunction and vascular stiffening (49). Iron overload also determines vaso-occlusive pulmonary disease with secondary pulmonary hypertension. Overall, vascular damage enhances heart dysfunction. Given high level of oxidative stress, antioxidant agents together with iron chelating agents is the rational therapy in these patients. Experimental studies suggest a cardiac protective role of taurine. A promising perspective is the use of Ca\(^{2+}\) channel blockers, especially of amlodipine, due to its coronary vasodilator and antioxidant action, but also the specific mechanism of iron action on L-type Ca\(^{2+}\) channels (50).

Even if the direct role of iron as a risk factor for cardiovascular disease is unclear, oxidative stress caused by catalytic iron is a certainty. Nature has effective weapons against this mechanism in physiological conditions, as is the formation of specific complexes (complex 1-Fe) with antioxidant role. The therapeutic use of this mechanism is considered by Gross's team as innovative as these complexes could form the basis of the future design of "intelligent antioxidants". The authors demonstrate the mechanism of these complexes, which are bipolar, that is they can bind to both types of lipoproteins, LDL and HDL. The consequence is the catalytic decomposition of harmful species, i.e. permanent and targeted replacement of oxidized environment, in contrast to current antioxidants which have limited effect on reactive oxygen species and can damage the arterial wall. An interesting remark of the study is that directing the bivalent ferrous iron to these complexes by specific enzymatic methods may increase the antiatherogenic effect of HDL-cholesterol (51).

REFERENCES


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