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REVIEW

The Role of Iron in the Pathogenesis of Atherosclerosis

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Summary

Ferritin and increased iron stores first appeared on the list of cardiovascular risk factors more than 30 years ago and their causal role in the pathogenesis of atherosclerosis has been heavily discussed since the early 1990s. It seems that besides traditional factors such as hyperlipoproteinemia, hypertension, diabetes mellitus, obesity, physical inactivity, smoking and family history, high iron stores represent an additional parameter that could modify individual cardiovascular risk. The role of iron in the pathogenesis of atherosclerosis was originally primarily associated with its ability to catalyze the formation of highly reactive free oxygen radicals and the oxidation of atherogenic lipoproteins. Later, it became clear that the mechanism is more complex. Atherosclerosis is a chronic fibroproliferative inflammatory process and iron, through increased oxidation stress as well as directly, can control both native and adaptive immune responses. Within the arterial wall, iron affects all of the cell types that participate in the atherosclerotic process (monocytes/macrophages, endothelial cells, vascular smooth muscle cells and platelets). Most intracellular iron is bound in ferritin, whereas redox-active iron forms labile iron pool. Pro-inflammatory and anti-inflammatory macrophages within arterial plaque differ with regard to the amount of intracellular iron and most probably with regard to their labile iron pool. Yet, the relation between plasma ferritin and intracellular labile iron pool has not been fully clarified. Data from population studies document that the consumption of meat and lack of physical activity contribute to increased iron stores. Patients with hereditary hemochromatosis, despite extreme iron storage, do not show increased manifestation of atherosclerosis probably due to the low expression of hepcidin in macrophages.

Key words

Iron • Iron stores • Oxidative stress • Inflammation • Macrophages • Atherosclerosis

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In 1981, Sullivan first proposed iron as a cardiovascular risk factor, suggesting that the lower incidence of cardiovascular disease (CVD) in premenopausal women compared to men and postmenopausal women could be explained by lower body iron stores. He hypothesized that iron depletion by regular phlebotomy may be protective (Sullivan 1981). Data from a prospective randomized study of 1,931 men from eastern Finland (Salonen *et al.* 1992) documented that in subjects with initial plasma ferritin concentration levels >200 μg/l myocardial infarction occurred 2.2 times more often than in men with ferritin levels <200 μg/l over a 5-year period. In a different study, Tuomainen *et al.* (1998) estimated body iron stores more precisely as the ratio of circulation transferrin receptor (TfR) to plasma ferritin concentrations, and showed that in men with higher stored iron (i.e. lower TfR/ferritin ratio) the risk of acute myocardial infarction was 2-3 times higher compared to a cohort with low iron stores over an average 6.4-year follow-up period. In the "Bruneck Study", Austrian authors observed a strong correlation between asymptomatic atherosclerosis in the carotid arteries and iron stores, both in men and women (Kiechl

et al. 1994). The same authors showed in a prospective study that initial serum ferritin was one of the strongest predictors of overall progression of atherosclerosis (Kiechl *et al.* 1997). We also demonstrated in a cohort of healthy men that body iron stores positively correlated with asymptomatic carotid atherosclerosis (Syrovátka *et al.* 2011). To prove the causal association of iron with CVD risk, several prospective studies evaluating the impact of changes in iron status have been performed. Kiechl *et al.* (1997) further proved that over a five-year follow-up lowering stored iron had a protective effect on the development of cardiovascular disease. On the other hand, iron accumulation increased the risk of coronary heart disease. Drüeke *et al.* (2002) observed in patients with end-stage renal disease that individuals receiving i.v. iron supplementation had higher plasma ferritin, higher intima-media thickness in the common carotid artery and higher plasma concentrations of advanced oxidation protein products (AOPP) compared to control subjects with no iron supplementation. On the other hand, two studies independently proved that lowering iron stores by regular blood donation resulted in a significant decrease in CVD risk (Meyers *et al.* 2002, Salonen *et al.* 1998). However, it should be noted that blood donors may be healthier than the general population. In addition, iron chelation in patients with coronary vascular disease has been shown to be associated with improved endothelial function (Duffy *et al.* 2001). The role of iron in the pathogenesis of atherosclerosis has always been associated with enhanced oxidative stress. Iron participates in a redox reaction through the transfer of electrons between the ferrous (Fe^{2+}) and ferric (Fe^{3+}) states and catalyses the formation of highly reactive oxygen species, namely the hydroxyl radical ($OH^{\cdot}$): $O_2^{\cdot-} + H_2O_2 \rightarrow O_2 + OH^- + OH^{\cdot}$.

The catalytic effect of iron is described in the Fenton reaction (Table 1).

Table 1. The Fenton reaction.

$Fe^{3+} + O_2^{\cdot-} \rightarrow O_2 + Fe^{2+}$
$Fe^{2+} + H_2O_2 \rightarrow OH^{\cdot} + OH^- + Fe^{3+}$

$O_2^{\cdot-}$ – superoxide, O_2 – molecular oxygen, OH^- – hydroxide anion, $OH^{\cdot}$ – hydroxyl radical.

Highly reactive hydroxyl radicals cause the oxidative modification of lipids and proteins as well as DNA damage, and are also responsible for the impairment of cell proliferation, endothelial dysfunction and immune system alterations.

Oxidative modification of lipoproteins, namely low-density lipoproteins (LDL), remains one of the crucial events in atherogenesis. In this process, both lipid and protein moieties are altered. Reactive oxygen species (ROS) are prone to attack the chains of fatty acids (FA) (especially unsaturated FA) in triglycerides, cholesteryl esters and phospholipids, leading to the formation of free and esterified peroxides (conjugated dienes), hydroxides, malondialdehyde (MDA) and other aldehydes, pentane and other hydrocarbons. Cholesterol itself is mostly oxidized into 7-ketocholesterol, cholesterol-β-epoxide and 7-β-hydroxycholesterol. The oxidation products of phospholipids include lysophosphatidylcholine and other molecules. Apoproteins are transformed into carbonyls or proteolysed fragments with certain modified amino acids (cysteine, cystine, histidine, methionine, lysine, arginine, tryptophan, tyrosine) and lipid-protein adducts, such as lipofuscins (Parthasarathy *et al.* 2010). Oxidized LDL (oxLDL) is involved in atherosclerosis on several levels, especially: 1) endothelial activation and dysfunction, 2) activation of macrophages and their transformation into foam cells, 3) activation of innate and adaptive immune responses.

Most data concerning LDL oxidation come from *in vitro* studies that use different pro-oxidative agents, including iron (Steinberg *et al.* 1989, Chait and Heinecke 1994, Fuhrman *et al.* 1994). Both ferrous (Fe^{2+}) and ferric (Fe^{3+}) iron have been shown to be effective LDL oxidizers, and oxidative modification can occur even in lysosomes (Satchell and Leake 2012). There is evidence that LDL oxidation is mostly mediated by the cells of the arterial wall. However, increased concentration of oxLDL has been found in the plasma of patients with acute myocardial infarction, unstable and stable angina pectoris, as well as in subjects with type 2 diabetes mellitus (Ehara *et al.* 2001, Njajou *et al.* 2009).

On the other hand, some studies have repudiated the association between iron stores and atherosclerosis (Frey *et al.* 1994, Auer *et al.* 2002, Waalen *et al.* 2002). Opponents of the "iron hypothesis" mainly question the role of iron as an independent cardiovascular risk factor, since iron positively correlates with other risk parameters, namely LDL-cholesterol. In one prospective study, reduction of iron stores by regular phlebotomy at 6-month intervals over a 6-year period had no effect on all-cause mortality or death or on non-fatal myocardial infarction or stroke in patients with peripheral vascular

disease. However, survival was significantly improved in subjects when iron reduction was initiated before the age of 60 (Zacharski *et al.* 2007). Since these results show a strong interaction with age, it may be speculated that lowering iron stores could be even more protective in the primary prevention of younger patients. The reason for the inconsistencies with data on iron is also connected to the differences and inaccuracies that arise when assessing stored iron (ferritin, transferrin, the TfR/ferritin ratio, etc.). Both ferritin and transferrin, which act as acute phase proteins, increase in patients with acute or chronic inflammation (Ahmed *et al.* 2012). Ferritin further increases in malignancies (both in solid tumors and leukemias) (Sackett *et al.* 2016).

Hereditary hemochromatosis (HFE) is a genetic disease characterized by iron overload due to increased iron resorption in the small intestine. Prevalence ranges from 1:200 to 1:400 in Caucasians of northern European ancestry. Over 90 % of clinically manifest patients are homozygotes (Tyr/Tyr) for the HFE gene polymorphism, Cys282Tyr (rs1800562). The other most common genetic variant is His63Asp (rs1799945) but has much lower penetrance (Whittington 2002). Thus, it is likely that carriers of the mutation in the HFE gene are at higher cardiovascular risk. Indeed, several authors report an increase in cardiovascular events among Cys282Tyr (rs1800562) heterozygotes (Roest *et al.* 1999, Tuomainen *et al.* 1999, Rasmussen *et al.* 2001). However, further studies, including one meta-analysis of 53,880 subjects, found no support for these findings (Ellervik *et al.* 2005, van der A *et al.* 2008, Engberink *et al.* 2010). The Rotterdam Study, which analyzed the frequency of both the HFE Cys282Tyr (rs1800562) and His63Asp (rs1799945) gene polymorphisms, showed no significant relation to stroke or carotid artery atherosclerosis (Njajou *et al.* 2002). Thus, iron overload in HFE is not associated with increased cardiovascular risk, despite the reported increased oxidative stress in these individuals (Broedbaek *et al.* 2009). Some reports even speculate about the possible protective effect of HFE (especially in homozygous patients) against atherosclerosis (Miller *et al.* 1994). Iron metabolism in hereditary hemochromatosis is characterized by increased iron resorption and excessive deposition in tissues, namely the liver, pancreas, heart and pituitary gland. Iron resorption, plasma iron concentration and tissue distribution are regulated by hepcidin and ferroportin. In duodenal enterocytes, ferroportin located on the basolateral membrane enables the transport of dietary iron to the blood. The peptide hepcidin directly binds to ferroportin, thus inhibiting the efflux of intracellular iron. Since the homozygous form of HFE is associated with hepcidin deficiency (Lee and Beutler 2009), lack of hepcidin results in the overexpression of ferroportin in the duodenum and increased iron resorption, which is associated with systemic iron overload in tissues. In macrophages, however, lack of hepcidin and increased ferroportin-mediated iron efflux lead to a decrease in intracellular iron levels (Valenti *et al.* 2011). Monocytes/ macrophages play a key role in the pathogenesis of atherosclerosis and the amount of iron within these cells may be of crucial importance. Thus, the lower levels of intracellular iron in the macrophages of HFE patients, even of homozygous form, seem to protect against atherosclerosis.

In β-thalassemia and sickle cell anemia (hereditary disorders characterized by the synthesis of abnormal hemoglobin and increased plasma iron turnover), iron-dependent oxidative stress injury is associated with frequent hemolytic events. Heme, when released into circulation, increases the level of redox-active iron. In addition, thalassemic patients suffer from iron overload due to increased iron absorption and regular erythrocyte transfusions. Cheung *et al.* (2002) demonstrated that patients with β-thalassemia had greater arterial stiffness and endothelial dysfunction than controls. In the ARIC Study, black patients with sickle cell trait had increased incidence of ischemic cerebrovascular stroke during a median follow-up of 22 years (Caughey *et al.* 2014).

Iron and transformation of monocytes/ macrophages into foam cells

Atherosclerosis is a chronic fibroproliferative inflammatory process characterized by thickening of the arterial intima and formation of atherosclerotic plaque. As described by Mallat *et al*. (2009), both innate and adaptive immunity are involved, resulting in atherosclerosis progression. Monocytes, the key cellular effectors of innate immunity, are attracted to activated endothelial cells by chemoattractants, e.g. macrophage chemoattractant protein-1 (MCP-1). Circulating monocytes adhere to the endothelial surface and become trapped by cytoadhesive molecules, e.g. vascular cytoadhesive molecule-1 (VCAM-1), intracellular adhesion molecule-1 (ICAM-1) or E-selectin. Subsequently, monocytes enter the subendothelial space

of the arterial intima, become activated by macrophage-colony stimulating factor (M-CSF) (produced by T-lymphocytes, endothelial cells and macrophages themselves) and differentiate into macrophages. Both pro-inflammatory M1 macrophages and anti-inflammatory M2 macrophages have been detected in atherosclerotic plaque (Butcher and Galkina 2012). M1 macrophages, driven by interferon γ (IFN-γ) and lipopolysaccharide (LPS), express on their surface scavenger receptors, through which oxidatively modified lipoproteins (oxLDL) are internalized. Macrophages then become overfilled with cholesteryl ester and transform into foam-cells. Moreover, experimental studies indicate that oxLDL in combination with β2-glycoprotein I (β2GPI) triggers the formation of immune complexes with specific IgG antibodies, which are subsequently absorbed by macrophages via the Fcγ fragment (Kajiwara *et al.* 2007). Pro-inflammatory M1 macrophages also induce smooth muscle cell (SMC) proliferation and their migration from media into the arterial intima. Atherosclerotic advanced unstable lesions, which are characterized by a relatively large lipid-rich necrotic core, predominantly contain M1 macrophages. In addition, M1 macrophages may further destabilize atherosclerotic plaque by producing matrix metalloproteases (MMP-1, MMP-3, MMP-9) *via* the hydrolysis of collagen fibers within the fibrous cap (Ley *et al.* 2011).

On the other hand, the role of M2 macrophages in atherogenesis is more complicated. IL1-β, IL-4, IL-10 and IL-13 (produced by Th2 lymphocytes and mast cells) transform monocytes into M2 macrophages, which are known to promote the fibrotic process through the recruitment of fibroblasts and the production of connective fibers (Gordon and Martinez 2010). By producing anti-inflammatory cytokines (especially IL-4, IL-10, IL-13), M2 macrophages have a major impact on the deactivation of endothelial cells, Th1 lymphocytes and other macrophage populations. M2 cells promote plaque stability by inducing the proliferation of SMC and the production of collagen I (Medbury *et al.* 2013). Even though both M1 and M2 subtypes are found in the fibrous cap, M2 macrophages are associated with plaque stabilization. Unlike M1 cells, M2 macrophages lack scavenger receptors and are thus incapable of internalizing oxidized LDLs. However, M2 macrophages are also capable of producing various MMPs (MMP-9, MMP-12, MMP-13, MMP-14), which are used for clearing apoptotic cells and plaque remodeling. The roles of other macrophage subsets are still poorly understood from an atherosclerosis prospective. These phenotypes include Mhem (hemorrhage-associated macrophages), Mox (macrophages activated by oxidized phospholipids) and M4 (macrophages induced by hemoglobin, oxidized phospholipids or chemokine ligand 4).

M1 macrophages have low iron turnover, low expression of ferroportin, heme oxygenase-1 (HMOX1) and CD163 (a scavenger receptor responsible for internalizing the haptoglobin-hemoglobin complex) and are rich in ferritin and prone to iron accumulation. M2 macrophages have, in contrast to M1 cells, an iron-release phenotype through the higher expression of ferroportin, HMOX1 and scavenger receptor CD-163 and the lower expression of ferritin. M2 macrophages exhibit higher iron turnover (both uptake and efflux) but lower intracellular iron status (Recalcati *et al.* 2010). Since high body iron stores are associated with increased oxidation stress and inflammation, it seems plausible that pro-inflammatory stimuli lead to the polarization of monocytes into the M1 (iron-rich) phenotype (Fig. 1). In our study of cultivated THP-1 monocytes, we demonstrated that iron-loading with human transferrin was associated with native LDL oxidation, increased accumulation of oxLDL and scavenger receptor stimulation (Kraml *et al.* 2005). Oxidation stress and inflammation are closely related processes and one can be induced by the other. Although inflammatory cells produce reactive species, reactive oxygen and nitrogen species can serve as signaling molecules, which enhance pro-inflammatory gene expression in tissues (Anderson *et al.* 1994, Flohé *et al.* 1997). Intracellular iron is predominantly stored in ferritin (Fe^{3+}). However, a certain amount exists as a cytosolic labile iron pool (LIP), which indicates that redox-active iron catalyzes the formation of ROS from both endogenous and exogenous sources. LIP and ROS are strongly interrelated, as documented by iron import and iron chelation studies (Yuan *et al.* 2004). In one *in vivo* study, LIP catalyzed ROS-mediated oxidation/peroxidation of LDL in macrophages, endothelial cells and VSMCs, a process that can be prevented by using the iron chelator, deferiprone (Matthews *et al.* 1997). Lapenna *et al.* (2007) showed that LIP, such as low-molecular-weight iron from *ex vivo* carotid endarterectomy specimens, significantly correlates with plasma ferritin and markers of lipid peroxidation. However, the relation between LIP and intracellular ferritin as well as plasma ferritin has not been fully clarified. In a recent clinical study of ours, we measured LIP in circulating monocytes and demonstrated

that patients with a history of cardiovascular events (CVE) had a significantly higher concentration of iron in their intracellular LIP than healthy controls and that LIP correlated with markers of atherosclerosis progression and arterial stiffness (Riško *et al.* 2017). Xiong *et al.* (2003) demonstrated in TNF-α and lipopolysaccharide (LPS) stimulated macrophages that intracellular label iron pool (iron not bound in ferritin) leads to IKK and NFκB activation through the toll-like receptor 4 (TLR4)-dependent signaling cascade (Xiong *et al.* 2003). The phagocytosis of erythrocytes in areas of intraplaque hemorrhage represents another mechanism of iron accumulation in macrophages (Yuan *et al.* 1996). However, more recent studies reveal that these macrophages represent a subtype distinct from M1 and M2 phenotypes (Boyle *et al.* 2011, Habib and Finn 2014). Macrophages in hemorrhage areas such as Mhem (hemorrhage-associated macrophages), which are similar to M2 macrophages, are characterized by high expression of CD163 (a receptor responsible for internalizing the haptoglobin-hemoglobin complex), ferroportin and HMOX1, making these cells prone to iron efflux. However, heme itself leads to the pro-inflammatory activation of M1 macrophages mediated by TLR-4 (Figueiredo *et al.* 2007). On the other hand, HMOX1 has significant atheroprotective effects due to its anti-inflammatory, anti-oxidant and anti-apoptotic properties.

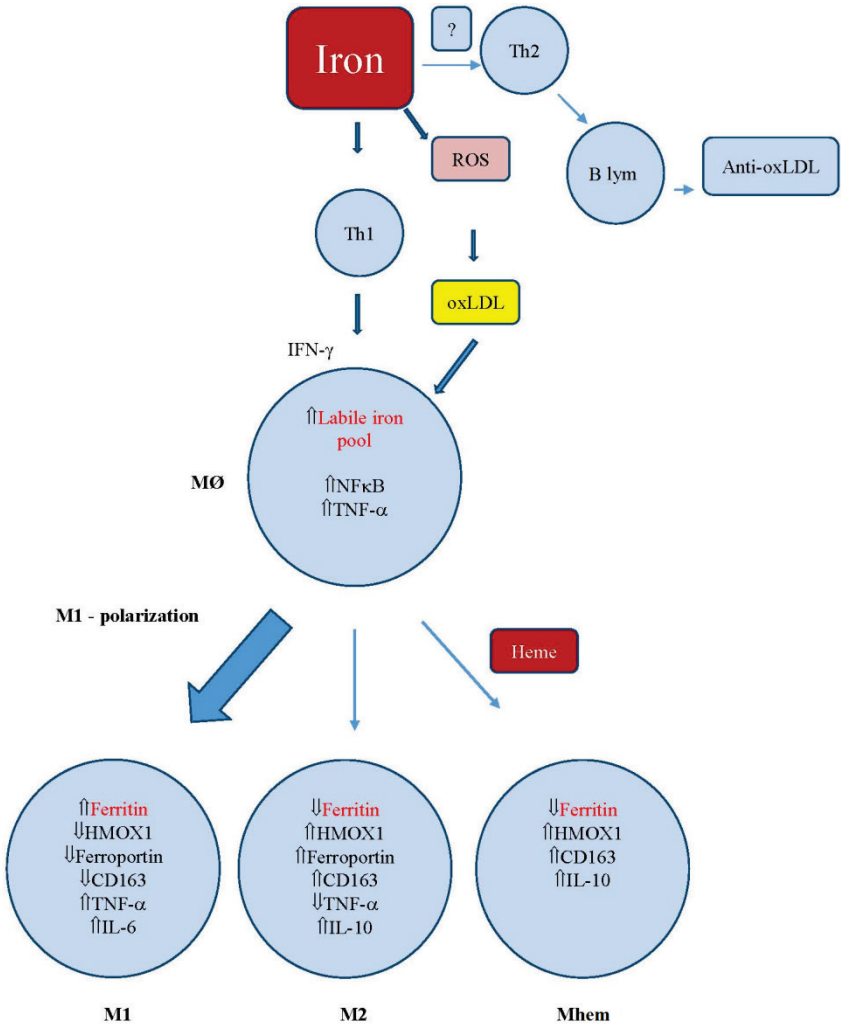

Fig. 1. Possible mechanism of iron influence on monocyte polarization.

Heme-oxygenase 1 plays one of the key roles in intracellular iron metabolism. This enzyme catalyses the degradation of heme into biliverdin, carbon monoxide and free iron, and subsequently converts biliverdin into bilirubin *via* bilirubin reductase. It has been shown that HMOX1 is upregulated by oxidative stress and various inflammatory signals and that through its immunomodulatory and anti-inflammatory properties it drives macrophages into the M2 phenotype (Naito *et al.* 2014). Both biliverdin and bilirubin serve as antioxidants

(Stocker *et al.* 1987) and carbon monoxide is a signal molecule with anti-inflammatory, anti-proliferative and vasodilatory properties. HMOX1 upregulates (directly or through carbon monoxide) the expression of anti-inflammatory interleukin 10 (IL-10) *via* the activation of phospho-p38 mitogen-activated protein kinase (p38MAPK). IL-10 itself also induces HMOX1 expression through the activation of signal transducer and activator of transcription 3 (STAT-3) and phosphatidylinositol 3-kinase (PI3K) (Naito *et al.* 2014). Of the two HMOX isoforms identified in humans – HMOX1 and HMOX2 – only HMOX1 is a stress-inducible enzyme. The gene expression of HMOX1 is regulated by various transcription factors, e.g. NFκB, hypoxia-inducible factor-1 and other signals such as mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K) cascades, depending on the character of the stressor and cell type (Paine *et al.* 2010). Iron released from heme by HMOX1 induces the expression of intracellular apoferritin, followed by the incorporation of iron into ferritin and the decrease of intracellular redox-active iron (Fe^{2+}) in the labile iron pool.

Ferritin is the main intracellular iron storage protein. Ferritin molecules are composed of heavy (H) and light chains (L) and the ratios of H and L subunits vary among different tissues. It has been demonstrated that some ferritin (both H and L chains) is released into plasma and that H-ferritin serves as a signal molecule and has an immunosuppressive function. The mechanism of ferritin secretion (H and L chains) and its origin are not fully understood. However, Wesselius *et al.* (1994) documented ferritin secretion in hepatocytes and Kupffer cells. Ferritin binds to certain subsets of myeloid cells and B and T lymphocytes *via* the specific receptor TIM-2 (T-cell immunoglobulin and mucin domain 2) and has an inhibitory effect on cell proliferation (Recalcati *et al.* 2008). In mature myeloid cells as well as in myeloid precursors, ferritin inhibits cell migration and differentiation. Thus, the anti-inflammatory properties of plasma ferritin together with the incorporation of intracellular redox-active iron into apoferritin may protect against atherosclerosis. However, hyperferritinemia as a marker of elevated iron stores has been associated with increased cardiovascular risk. Higher oxidative stress and immune system activation associated with iron overload indicate the dysregulation of antioxidant and anti-inflammatory mechanisms in these individuals.

Oxysterols, products of the enzymatic and non-enzymatic oxidation of cholesterol, have also been shown to possess immunomodulatory properties. These bioactive molecules further regulate cholesterol and sphingolipid metabolism, platelet aggregation and apoptosis through interaction with specific sensors, e.g. liver X receptors (LXR) and oxysterol-binding protein (OSBP). Liu *et al.* (1997) documented that 25-OH-cholesterol, 7β-OH-cholesterol and other oxysterols stimulate the production of IL-8 by human monocytes and macrophages isolated from atherosclerotic plaque. On the other hand, oxysterols in macrophages interfere with NFκB *via* LXRs, resulting in the inhibition of TLR-inducible inflammatory molecules, e.g. IL-1β, MCP-1 (monocyte chemoattractant protein-1) and iNOS (inducible nitric oxide synthase) (Joseph *et al.* 2003).

Iron, endothelial cells, vascular smooth muscle cells and thrombocytes in atherogenesis

TLR-4-dependent signaling pathways seem to play a key role in atherosclerosis progression. Besides macrophages, TLR-4 is expressed in other cell types involved in atherosclerosis (endothelial cells, thrombocytes).

In endothelial cells TLR-4 activation upregulates the expression of chemoattractant and cytoadhesive molecules. Upon the administration of iron in healthy individuals, which induces the generation of superoxide anions in whole blood and endothelial dysfunction, a direct association has been reported between iron excess and endothelial dysfunction. Balla *et al.* (1990) demonstrated in an *in vitro* study that iron-loaded cultured endothelial cells that use iron-chelating fungistat 8-hydroxyquinoline are associated with cytotoxicity accompanied by membrane lipid peroxidation. In another study, cultivated human umbilical vein endothelial cells (HUVEV) loaded with non-transferrin-bound iron led to an increase in the intracellular labile iron pool and subsequent endothelial dysfunction associated with the induction of the adhesion molecules, VCAM-1, ICAM-1 and selectin (Kartikasari *et al.* 2004). This team further documented that iron-loaded endothelial cells exhibit enhanced activation and production of adhesion molecules in response to Cytomegalovirus (CMV) or Chlamydia pneumoniae (CHP) infection (Kartikasari *et al.* 2006). It has been suggested that increased intracellular iron may facilitate an endothelial response to various stimuli associated with the initiation and progression of atherosclerosis (namely, oxidized LDL), even though the causal roles of specific infectious agents,

such as CMV and CHP, are no longer considered plausible. As demonstrated by experimental studies, oxLDL leads to the dysfunction of endothelial cells through the activation of PKC, MAPK and other signaling pathways (Ren *et al.* 2000, Li *et al.* 2003). Cominacini *et al.* (2000) proved in bovine endothelial cells that oxLDL activates transcription factor NFκB *via* the enhanced intracellular production of free radicals.

Vascular smooth muscle cells (VSMC) in arterial media are also largely regulated by TLR-4. Under TLR-4-dependent activation, these cells proliferate and migrate into the intima (Pasterkamp *et al.* 2004). This process is accompanied by a phenotype change from the contractile to the synthetic, which produces an extracellular matrix rich in collagen leading to the formation of a fibrous cap. As documented by several *in vitro* studies, there is close cooperation between macrophages and VSMCs. Macrophages control VSMC activation *via* the production of IL-6 and platelet-derived growth factor (PDGF) (Zhu *et al.* 2000, Morisaki *et al.* 1992). It is reasonable to assume that intracellular iron in M1 macrophages indirectly promotes the activation of VSMCs in atherosclerosis. Moreover, iron chelation with desferrioxamine (DFO) significantly inhibited VSMC proliferation in one *in vitro* study (Wong *et al.* 2012).

Increased activation of thrombocytes *via* TLR-4 can lead to the formation of thrombi on the surface of unstable plaque (Jayachandran *et al.* 2010). In addition, changes in redox status substantially alter platelet activity. It has been documented that thrombocytes are capable of ROS production and that superoxide anions derived from platelets and other vascular sources promote platelet aggregation (Handin *et al.* 1977). As demonstrated by Praticò *et al.* (1999) in isolated human platelets, iron (Fe^{2+}) can directly stimulate aggregation through hydroxyl radical ($OH^{\cdot}$) formation and protein kinase C (PKC) activation. DFO may inhibit collagen-induced aggregation in this model.

Iron stores, dietary iron and physical activity

The recommended dietary iron intake in men and postmenopausal women is 8 mg/day, and in premenopausal women 18 mg/day (in pregnancy 27 mg/day and during lactation 9 mg/day (Institute of Medicine, Food and Nutrition Board 2001). In population studies, concentrations of plasma ferritin are used for assessing iron stores in the body. The reference interval in adults is 30-400 μg/l (men, aged 20-60 years), 15-150 μg/l (women, aged 17-60 years). In general, serum ferritin in adults between 20-50 years of age ranges from 15-300 μg/l (Association for Clinical Biochemistry 2012). In a cohort of Finnish men, the risk of coronary heart disease was significantly associated with iron intake. For each milligram of consumed iron, there was a 5 % increase in CHD risk (Salonen *et al.* 1992). Other studies that evaluate dietary iron intake with respect to CHD show rather conflicting data, but most of these reports do not analyze the iron source. It later became obvious that the problem is associated with heme iron intake and the consumption of meat and meat products. Heme iron from hemoglobin and myoglobin represents 10-15 % of the total iron intake in meat-eating populations, but over 40 % of absorbed iron is heme iron due to its higher bioavailability (Carpenter *et al.* 1992). As shown by Snowdon *et al.* (1984), there was a 60 % increase in the risk of fatal coronary events among men who consumed meat 6 times a week compared with men who consumed meat less than once a week. Increased risk of non-fatal myocardial infarction or fatal ischemic cardiac events with elevated heme iron intake are further supported by other studies (Ascherio *et al.* 1994, Klipstein-Grobusch *et al.* 1999). Risk estimates from pooled analyses and meta-analyses based on at least six cohorts document a significant 24 % increase in cardiovascular mortality when 50 g of processed meat is consumed daily (Wolk 2017). In a study by Micha *et al.* (2012) a daily intake of 50 g of processed meat was associated with a 30 % higher rate of CVD (RR=1.30; 95 % CI=1.17-1.45). Bioavailability of dietary iron, which comes from hemoglobin and myoglobin in meat, is much higher than from other sources since the absorption of heme iron is not inhibited by negative feedback in response to plasma ferritin, as distinct from the absorption of non-heme iron (Hunt *et al.* 2000). Iron from animal sources contributes to only 10-15 % of total dietary iron. However, over 40 % of absorbed iron is represented by heme iron (Hurrell and Egli 2010) and its absorption is further facilitated by animal proteins. Iron absorption inhibitors, namely phytates, polyphenols, soy proteins, casein, whey and egg white, only decrease non-heme iron absorption. Calcium was believed to have an inhibitory effect on both non-heme and heme iron absorption (Cook *et al.* 1991, Hallberg *et al.* 1991). However, in one study, the addition of 200 mg of calcium to a maize-based diet had no such effect (Troesch *et al.* 2009) (Table 2 and Table 3).

Table 2. Iron absorption inhibitors.

Name	Dietary source
Phytate	Cereals, grains
Polyphenols	Fruit, vegetables, cereals, legumes, tea, coffee, wine
Soy proteins	Soybean
Calcium	Milk products
Casein, whey	Milk
Egg white	Eggs

Table 3. Iron absorption enhancers.

Name	Dietary source
Ascorbic acid	Fruit, vegetables, supplements
Muscle proteins	Pork, beef, chicken, fish

Another discussed mechanism that contributes to iron overload seems to be low physical activity. Lauffer *et al.* (1981) outlines the possible mechanisms by which physical training promotes iron excretion. These include a) iron losses by sweating, b) gastrointestinal tract losses due to relative intestinal ischemia or stress gastritis, c) intravascular hemolysis with hemoglobinuria due to mechanic or osmotic destruction of erythrocytes, d) early release of iron from transferrin due to intermittent relative acidosis and e) increase of muscle mass associated with the enhanced incorporation of iron into myoglobin (Lauffer *et al.* 1991).

Thirty-five years ago in his iron hypothesis, Sullivan (1981) proposed that lower ferritin in premenopausal women due to monthly iron loss has a protective effect against atherosclerosis. The incidence of coronary heart disease and stroke in premenopausal women is indeed lower than in men of the same age and the risk increases after the menopause. Moreover, the Framingham Heart Study and the Multi-Ethnic Study of Atherosclerosis (MESA) showed that early menopause increases cardiovascular events (Lisabeth *et al.* 2009, Wellons *et al.* 2012). The lower CVD risk in premenopausal women has been sometimes attributed to the direct effect of estrogens. However, the results of MESA found no association between plasma estradiol and markers of subclinical atherosclerosis – intima-media thickness (IMT) or the coronary calcium score (Ouyang *et al.* 2009).

Lower iron, lower hemoglobin and CVD risk

Not only iron overload but also iron deficiency and chronic anemia are associated with increased cardiovascular risk, which suggests that the relationship between iron and CVD is not linear but rather U-shaped (Shoji *et al.* 2016). In one prospective cohort of the ARIC Study, the presence of anemia (defined as a hemoglobin concentration <130 g/l in men and <120 g/l in women) was independently associated with increased risk of CVD (HR 1.41 [95 % CI: 1.01, 1.95]) (Sarnak *et al.* 2002). Results from the "Ludwigshafen Risk and Cardiovascular Health Study" showed that OR for CAD in the lowest quartiles of hemoglobin was 1.40 (95 % CI: 1.04, 1.90) compared to the highest gender-specific quartiles when adjusting for iron and ferritin. The authors suggested that both hemoglobin levels and iron status have an independent impact on CVD risk (Grammer *et al.* 2014). The association between anemia and CVD risk has traditionally been seen in terms of the worsening of myocardial ischemia. In addition, chronic anemia may lead to ventricular remodeling and heart failure. Increased cardiac output and high sympathoadrenal activity caused by anemia may result in left ventricular hypertrophy. Anemia further accompanies other CVD risk factors such as chronic kidney disease (CKD), chronic inflammatory processes and low nutritional status. Apart from oxygen transport, iron is involved in mitochondrial function, the metabolism of lipids and proteins and DNA synthesis. Iron depletion may thus lead to the impaired function of different tissues, namely the central nervous system, muscle tissue, the myocardium, the immune system and the thyroid gland. However, most patients with depleted iron stores develop anemia and, therefore, the question whether iron deficiency might contribute to atherosclerosis irrespective of hemoglobin concentrations remains unclear.

Conclusion

In addition to traditional risk factors, such as hypertension, hyperlipoproteinemia, diabetes, obesity, lack of physical activity and smoking, iron seems to play an important role in the pathogenesis of atherosclerosis, as documented by both observational and experimental studies. Iron mediates the oxidative modification of lipoproteins and through its pro-inflammatory properties impacts the initiation, progression and destabilization of atherosclerotic plaque. In patients where high body iron

stores represent an additional cardiovascular risk factor, limiting meat consumption and increasing physical activity seem plausible countermeasures and should be added to the list of traditional "healthy life-style" recommendations. However, further research is required in order to formulate guidelines for an exact "iron diet", sufficient physical activity and, potentially, iron chelation, especially in patients at high cardiovascular risk and with elevated body iron stores.

Conflict of Interest

There is no conflict of interest.

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