**Ironing out macrophages in atherosclerosis**

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<th>Journal:</th>
<th>Acta Biochimica et Biophysica Sinica</th>
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<td>Manuscript ID</td>
<td>ABBS-2022-373.R3</td>
</tr>
<tr>
<td>Manuscript Type:</td>
<td>Review</td>
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<tr>
<td>Date Submitted by the Author:</td>
<td>15-Dec-2022</td>
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<td>Complete List of Authors:</td>
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<tr>
<td>Keywords:</td>
<td>atherosclerosis, iron, macrophage</td>
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Review

Ironing out macrophages in atherosclerosis

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Running title: Ironing out macrophages in atherosclerosis

Received: 3Jul2022
Accepted: 25Aug-2022

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Abstract
The most common cause of death worldwide is atherosclerosis and related cardiovascular disorders. Macrophages are important players in the pathogenesis of atherosclerosis and perform critical functions in iron homeostasis due to recycling iron by phagocytosis of senescent red blood cells and regulating iron availability in the tissue microenvironment. With the growth of research on the “iron hypothesis” of atherosclerosis, macrophage iron has gradually become a hotspot in the refined iron hypothesis. Macrophages with the M1, M2, M(Hb), Mox, and other phenotypes have been defined with different iron-handling capabilities related to the immune function
and immunometabolism of macrophages, which influence the progression of atherosclerosis. In this review, we focus on macrophage iron and its effects on the development of atherosclerosis. We also cover the contradictory discoveries and propose a possible explanation. Finally, pharmaceutical modulation of macrophage iron is discussed as a promising target for atherosclerosis therapy.

**Keywords:** atherosclerosis; iron; macrophage

1. **Introduction**

Atherosclerotic cardiovascular disease is a serious global health concern and one of the leading causes of mortality[1, 2]. Atherosclerosis is caused by the internal retention of cholesterol-rich and apolipoprotein B-containing lipoproteins in vulnerable parts of the arterial blood arteries, resulting in persistent inflammation[3]. Lipoproteins sequestered in the artery wall are subjected to a range of modifications (including enzymatic and nonenzymatic cleavage, oxidation, and aggregation), triggering a proinflammatory environment of the overlying endothelium. Although many cell types, such as endothelial cells, monocytes, and vascular smooth muscle cells, play roles in developing atherosclerotic plaques, macrophage differentiation and retention within the arterial wall are indispensable conditions for atherosclerosis[4]. The recruitment and entrance of monocyte-derived cells into the subendothelial region are responsible for the immunological response. They differentiate into mononuclear phagocytes, so-called macrophages, to consume the accumulated modified lipoproteins, transforming them into cholesterol-loaded “foam cells” [4].

In almost every organism, iron is an essential trace element[5]. The chemical characteristics of iron as a transition metal account for its biological significance. Iron easily converts between Fe(II) and Fe(III) oxidation states in one-electron oxidation–reduction processes for complicated biological activities. However, excess iron can be toxic by catalyzing the Fenton reaction, converting hydrogen peroxide to hydroxyl radicals[6].

In 1981, Dr. Jerome Sullivan proposed the “iron hypothesis”, based on the fact that the incidence of cardiovascular diseases is higher in men than in premenopausal women and that the incidence goes up to the levels in the female gender when periodic blood loss stops in postmenopausal women[7, 8]. This hypothesis brought research to focus on elucidating the role of iron in the progression of atherosclerotic
plaques. Numerous animal experiments and clinical studies have provided evidence that iron plays a role in the formation of atherosclerosis, while others have offered contradictory data in this respect. Significantly, there is no increased risk of atherosclerosis in patients with hereditary hemochromatosis with severe lifelong iron overload, which is considered a "paradox" and a phenomenon difficult to explain by the iron hypothesis[9, 10].

With further research, the role of macrophage iron in atherosclerosis has attracted increasing attention and can explain this “paradox”[11]. Macrophages are also crucial for maintaining systemic iron homeostasis. Therefore, this article reviews the effect of macrophage iron on the progression of atherosclerosis.

2. Iron Homeostasis and Macrophages

Iron chemistry is associated with many essential biological processes required for life to exist[5, 12]. Iron is incorporated into proteins in mammalian cells, which are involved in oxygen transfer, cell respiration, DNA synthesis and repair, protein synthesis, etc. The physiological importance of iron can be demonstrated by clinical manifestations caused by iron deficiency and iron overload, including cognitive developmental disorders, congenital disabilities, and cardiovascular diseases[13]. Iron overload is implicated in several metabolic disorders, such as hemochromatosis, liver cancer, and neurodegenerative diseases[6]. Because the Fenton reaction triggered by free iron causes reactive oxygen species (ROS) production, iron overload may result in DNA, protein, and lipid damage or even cell death[6]. Thus, iron content must be accurately balanced at the cellular and systemic levels by tweaking the regulatory system to maintain iron homeostasis.

2.1 Systemic iron homeostasis

Iron is absorbed from the diet by duodenal enterocytes via divalent metal transporter 1 (DMT1) on the apical surface after Fe$^{3+}$ is reduced to Fe$^{2+}$ by Fe-reductase (DcytB). The hepcidin–ferroportin (FPN) axis controls whether the absorbed iron enters circulation (Figure 1). Hepcidin is a 25-amino-acid peptide released by hepatocytes that circulates in the bloodstream[14]. Hepcidin production is induced by systemic iron overload and inflammatory stimulation, whereas it is suppressed by iron deficiency, hypoxia, and erythropoiesis[14]. Therefore, when iron deficiency and erythropoietic signals are activated, the expression of hepatic hepcidin is reduced,
promoting iron intestinal absorption from the diet into the bloodstream via FPN. When body iron levels are elevated or infection is present, hepcidin expression increases. FPN, as the only known iron exporter, binds to hepcidin for internalization and degradation to limit FPN-mediated iron efflux and iron availability for pathogenic microorganism growth [14].

After entering circulation, iron is available for erythropoiesis or other physiological purposes. It is bound to the iron carrier transferrin (Tf) and taken up by transferrin receptor 1 (TfR1, also known as CD71)-mediated endocytosis. Cellular iron is utilized as a cofactor itself, heme, or iron-sulfur (Fe-S) clusters for many proteins or enzymes[14]. However, the daily demand for iron mainly depends on reticuloendothelial macrophages to phagocytose senescent red blood cells (see below).

2.2 The role of macrophages in iron homeostasis

Macrophages, the critical iron-handling cells in mammals, are present in all tissues and play a role in tissue homeostasis[13]. Macrophages may take up iron directly by iron transporters. The direct uptake of iron, also called extracellular non-Tf-bound Fe\(^{2+}\) iron, is mediated via plasma membrane-localized DMT1, which imports iron into the cytosolic labile iron pool (LIP)[15]. Macrophages mainly take up iron-containing molecules via receptors such as TfR1, LDL (low-density lipoprotein)-related receptor 1 (LRP1; also known as CD91), and the hemoglobin-haptoglobin receptor (CD163). Moreover, macrophages may phagocytose senescent erythrocytes and other apoptotic cells[13].

Plasma Fe\(^{3+}\) is mostly arrested by apo-Tf as holo-Tf to be endocytosed after binding with TfR1. Within the endosomal compartment, Fe\(^{3+}\) is reduced to Fe\(^{2+}\) at low pH by the prostate enzyme’s six-transmembrane epithelial antigen (STEAP3) before being released into cytosolic LIP via the assistance of DMT1[15] (Figure 1). Notably, macrophages activate the expression of heme oxygenase 1 (HO-1) after phagocytosis to breakdown heme-iron into Fe\(^{2+}\), blending into cytosolic LIP, and two anti-inflammatory products, biliverdin and carbon monoxide. Cytosolic LIP is destined for storage, export, incorporation with proteins or enzymes, or further trafficking into other organelles (Figure 1). In general, if not metabolized or exported, two main destinations of cellular iron in macrophages are ferritin for storage and mitochondria for biogenesis of heme and Fe-S clusters, two essential cofactors that assist with
electron transport and enzyme catalysis[16]. The capacity of ferritin to store iron is enormous, with up to 4500 atoms of iron caging in ferritin light (FtL) and ferritin heavy (FtH1) complexes[13]. The hepcidin-Fpn axis regulates the stored iron in macrophages for systemic iron demand. Most likely, more transporters exist to fulfil fine-tuning homeostasis because macrophage-conditional knockout of Fpn does not generate severe anemia in mice[17].

2.3 Cellular iron homeostasis of macrophages

Similar to other cell types, the iron regulatory protein (IRP)/iron-responsive-element (IRE) system also regulates macrophage iron homeostasis posttranscriptionally. IRP 1 or 2 (IRP1 or IRP2) recognizes and interacts with a conserved cis-regulatory hairpin IRE structure located in the 5′ or 3′ untranslated regions (UTRs) of target mRNA transcripts[14]. IRP binds to the 5′ UTR IREs of mRNAs encoding iron storage (ferritin H- or L-chain) and export (FPN) molecules in response to iron starvation signaling, inhibiting their translation. In contrast, IRP prevents mRNA degradation and enhances its stability by binding to multiple 3′ UTR IREs of the TfR1 transcript. As a result, the IRE-IRP system reduces the expression of molecules involved in iron export and storage while increasing the mRNA stability of the receptor involved in cellular iron uptake under iron depletion conditions[14]. IRP-IRE regulation is not supposed to be significant in iron-recycling reticuloendothelial macrophages because Tf-TfR1 is not a major iron-uptake pathway in these macrophages.

3. Atherosclerosis and the “Iron Hypothesis”

3.1 “Ironing out” atherosclerosis

Cardiovascular diseases (CVDs), such as myocardial infarction, stroke, and atherosclerotic occlusive diseases, are primarily caused by atherosclerosis[2]. The pathophysiology of atherosclerosis is characterized by a chronic inflammatory response with lipid accumulation and infiltration of monocyte-derived macrophages[4]. Several factors can contribute to the development of atherosclerosis, such as smoking, diabetes, hypertension, and high cholesterol[18]. In addition to treating diabetes and hypertension, intensive control of plasma lipid concentrations is considered part of primary and secondary CVD prevention[19].

A large number of randomized clinical trials have shown that using 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase inhibitors (such as statins) to reduce
detrimental lipid (LDL-C) levels will help prevent CVD[20, 21]. However, some patients fail to achieve LDL-C target levels with just statins in clinical practice. Furthermore, cardiovascular events occur occasionally even when LDL-C, diabetes, and hypertension are appropriately controlled[22]. Nevertheless, it is urgently necessary to develop and evaluate alternative local-targeting lipid-reducing medicines to prevent residual CVD risk.

Oxidative stress and inflammation have attracted great attention in the development of atherosclerosis in the past three decades. The opportunity to inhibit inflammation and oxidative stress sounds like a potential method of preventing and treating atherosclerosis[23-25]. However, inflammation is usually necessary to avoid infection, and anti-inflammatory medications have a narrow therapeutic window to treat atherosclerotic disease[26]. Therefore, new approaches are required to specifically target the inflammatory processes that occur with atherosclerosis. As iron regulates the body's inflammatory response[27], modulation of iron metabolism may be a choice to help effectively prevent atherosclerosis.

3.2 The “iron hypothesis”

Based on results from the Framingham study[28], the "iron hypothesis" proposed that iron is a critical factor in promoting cardiovascular diseases, which correlates with the significant sex difference[7]. This theory has been continually debated for more than 40 years. The effects of iron have been corroborated in a few animal models, and iron chelation mainly benefits these preclinical studies[29]. Further support for the iron hypothesis was given by the failure of estrogen replacement therapy in postmenopausal women[30]. Our recent unpublished data support the role of iron in the failure of estrogen replacement therapy. Human atherosclerotic lesions with redox-active iron and high levels of FtH and FtL expression indirectly support the iron hypothesis[31-33]. The iron content in lesions is directly correlated with cholesterol level[34]. Elevated iron level also exacerbates hypertriglyceridaemia in animal models, including zebrafish and rats[35, 36]. Likewise, iron-loaded patients with hereditary hemochromatosis show elevated hypertriglyceridaemia[37], which can be improved by therapeutic phlebotomy[38]. Recent genetic and epidemiological studies have demonstrated that triglycerides and triglyceride-rich lipoproteins are the main causal risk factors for residual atherosclerotic CVD[39]. Moreover, iron accumulation and ferritin production can be found in endothelial cells, macrophages, and vascular
smooth muscle cells within the plaque[11, 31].

From subsequent human studies, conflicting epidemiological evidence was presented regarding the role of iron in atherogenesis. The iron hypothesis suggests that iron promotes atherosclerosis development and progression and damages the cardiovascular system. Iron is thought to participate in generating reactive oxygen species and promoting lipid peroxidation, as well as activating endothelial cells, smooth muscle cell proliferation, and macrophage activation[10]. On the other hand, in patients with hemochromatosis as life-long iron overload, the incidence of atherosclerosis decreases rather than increases, which is thought to be the most convincing evidence against the "iron hypothesis"[9, 10].

The plausible explanation may come from macrophage iron depletion, promoted by low hepcidin through Fpn-mediated iron efflux from macrophages in hemochromatosis. Antiatherogenic and anti-inflammatory properties are likely to be present in “iron-depleted” or “iron fast-efflux” macrophages. Reducing macrophage iron content might be necessary to counteract the atherosclerotic effects of elevated systemic iron and decrease CVD susceptibility in hemochromatosis patients. Consistent with this view, our macrophage-conditional Fpn-knockout mice developed significantly severe atherosclerosis, and systemic iron chelation was sufficient to attenuate the aggravation of atherosclerosis[33]. As macrophages play a crucial role in atherosclerosis, selective iron deficiency in macrophages would be a new therapeutic mechanism for preventing foam cell formation and progression of atherosclerosis.

4. Macrophage Phenotypes and “Iron-status” in Atherosclerosis

The development of atherosclerosis is caused by an imbalance in lipid metabolism and an adverse immune response triggered by the recruitment of monocytes and their differentiation into cholesterol-laden macrophages in the artery wall[4]. These macrophages are exposed to inflammatory microenvironments that impact phenotypic differentiation and activation, such as oxidized lipids and cytokines[40]. Understanding the functions and mechanisms of macrophages' diverse phenotypes provides a comprehensive overview of the development of atherosclerotic plaques. However, there are still unresolved debates. Here, we focus on the iron status of the differential subtypes of macrophages in atherosclerotic plaques and their various functional roles based upon microenvironments such as lipid, intraplaque hemorrhage, and plaque regression.
Macrophages are classified into two groups: classical M1 (proinflammation) and alternative M2 (antiinflammation), which are activated by T-helper 1 and T-helper 2 cytokines, respectively. M1 macrophages produce proatherosclerotic cytokines such as IL-6, IL-1β, and tumor necrosis factor (TNF), which help maintain the inflammatory response[40]. M1 macrophages also produce ROS, which might aggravate plaque oxidative stress[40]. Oxidized low-density lipoprotein (oxLDL) is absorbed via scavenger receptors (such as CD36) on the surface of M1 macrophages and can convert into foam cells in response to lipid deposition, which is a vital stage in atherogenesis. As a result, M1 macrophages exhibit a proatherogenic character, with decreased FPN and HO-1[29]. M2 macrophages release anti-inflammatory cytokines such as IL-10 and have higher essential expression of mannose receptor (CD206), CD163, and arginase I than M1 macrophages. In addition, M2 macrophages do not convert into foam cells and have lower intracellular lipid levels than M1 macrophages[40]. In cellular and mouse studies, recent evidence suggests that M2 macrophages play a role in atherosclerosis regression, while their relevance in humans is unknown[41-43]. The characteristics of M1 and M2 macrophages match the cellular iron status. M1 macrophages have a high ferritin content and are susceptible to iron deposition, while M2 macrophages can metabolize and export iron, leading to reduced intracellular iron level[13] (Figure 2).

In addition to M1 and M2 macrophage phenotypes, several other macrophage populations are present in atherosclerotic lesions, such as Mox and M (Hb)[40]. Macrophages exposed to oxidized phospholipids are converted into particular Mox, which are marked by decreased phagocytic activity and chemotaxis and increased expression of redox regulatory genes induced by nuclear factor erythroid 2-related factor 2 (Nrf2)[44]. This type of macrophages increase the expressions of the oxidative stress enzymes HO-1 and hepcidin in response to oxLDL stimulation, resulting in iron deposition[45]. It was observed that oxLDL induces the activation of the Toll-like receptor 4 (TLR4)/nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway, which induces the autocrine synthesis of hepcidin[46]. The TLR4 pathway is activated by hepcidin-mediated iron deposition, which forms a vicious cycle with oxLDL. Furthermore, macrophage endoplasmic reticulum stress, the common pathophysiological mechanism in advanced atherosclerosis, can further increase hepcidin autocrine secretion[47].

Intraplaque hemorrhage, common in human advanced plaques, leads to the
release of free hemoglobin (Hb). This free Hb is ingested by the CD163 receptor by binding haptoglobin (Hp), which causes differentiation of classic M1 macrophages into M (Hb) or Mhem macrophages[48]. Heme is degraded by HO-1 to release free Fe$^{2+}$. FPN is upregulated by free iron in M (Hb) macrophages, reducing intracellular free iron levels. Therefore, M (Hb) is characterized by reduced iron content, decreased intracellular ROS production and inflammatory cytokine release, and resistance to foam cell formation with higher levels of FPN and the Hp–Hb scavenger receptor CD163[49]. Based on these characteristics, M (Hb) macrophages are supposed to be atheroprotective. However, in the context of intraplaque bleeding, M (Hb) macrophages were proposed to have a proatherogenic relationship with regions of intraplaque angiogenesis and permeability (Figure 2)[49, 50]. The inconsistency needs to be further investigated to determine whether the distinct consequences come from the different genetic backgrounds.

5. The Roles of Macrophage Iron

5.1 The effect of iron on macrophage polarization

As mentioned above, iron homeostasis and the expressions of iron-regulated genes strikingly shift during macrophage polarization. Macrophage iron management and immunological functions are related, and intracellular iron levels modulate macrophage polarization directly[29, 51]. Although several studies have reported inconsistent conclusions regarding the effect of iron on inflammation (review in [52]), the majority of studies show that increasing intracellular iron causes the proinflammatory M1 phenotype[53, 54]. The mechanisms whereby iron mediates polarization include: (i) modulation of cellular signaling pathways (NF-κB, MAPK, ROS generation, and so on); (ii) the shift of cellular metabolism; and (iii) epigenetic regulation/modification (reviewed in [55]). In view of iron metabolism, the change in intracellular iron level is involved in all aspects of the mechanisms, mainly concerning the production of cytokines. Fpn-deficient macrophages showed intracellular iron accumulation and increased expression of inflammatory cytokines[17, 33]. In mice with macrophage-specific inactivation of Fpn, wound healing is delayed, and macrophages tend to polarize to an M1 phenotype, resulting in prolonged inflammatory responses[56]. In contrast, enhanced iron efflux in macrophages mediated by hepcidin deficiency protects against atherosclerosis, suggesting that atheroprotective M2 polarization is induced by iron efflux (Figure
5.2 Catalytic effect of iron and links to lipoprotein metabolism

Iron is a potent catalyst and redox-active metal. The Fenton reaction in mammalian cells generates ROS toxicity by transferring electrons between the ferrous and ferric states[6]. One of the critical events in atherogenesis is lipoprotein oxidation. Iron retention promotes LDL oxidation and induces inappropriate cholesterol flux by increasing CD36-mediated cholesterol uptake and reducing ABC transporter ABCA1/ABCG1-mediated cholesterol transport. These lipid fluxes are regulated by CYP27A1 and liver X receptor α (LXRα) signaling, ultimately accelerating the formation of foam cells (Figure 2)[10, 33, 46, 58, 59]. Iron also increases the activation of TLR4-dependent macrophages by regulating the transport of TLR4 to cellular lipid rafts[60]. ROS and TLR4 signaling pathways are crucial in iron-driven foam cell formation and inflammatory activation. TLR4 inhibitors, iron chelators, and antioxidants suppress macrophage M1 phenotypic shift and cholesterol mismanagement[61, 62].

5.3 The role of macrophage iron in angiogenesis

M(Hb) macrophages were previously classed as anti-inflammatory and atheroprotective because of their enhanced cell iron effluence, cholesterol handling skills, and anti-inflammatory qualities[48]. However, iron reduction promotes the stability of hypoxia-inducible factor 1α (HIF1α) and the production of hypoxia-regulated vascular endothelial growth factor (VEGF) in M(Hb) macrophages. Macrophage VEGF production is linked to vascular permeability, intraplaque angiogenesis, and the recruitment of inflammatory cells, all of which contribute to severe atherosclerosis[50]. As a result, while macrophages have protective features such as enhancing iron and cholesterol efflux, they may activate other negative mechanisms (Figure 2). In addition, the iron efflux of macrophages may increase the iron level in the plaque microenvironment, potentially promoting oxidative stress in different types of cells. Further study must address whether the new angiogenesis can remove the exported iron. Nevertheless, a narrow manipulatable microenvironment is a highly complex challenge.
5.4 The role of iron in macrophage immunemetabolism

Inflammation and metabolism are inseparable, and the metabolic phenotype of macrophages can determine their inflammatory phenotype and vice versa. The changes in a metabolic pathway not only support the specific immune response of distinct macrophage subsets but also affect the specific function of macrophages and the progression of atherosclerosis.

The cellular iron status can affect immune metabolism differently in different macrophage subsets. The enzyme inducible nitric oxide synthase (iNOS), induced by TLR activation, produces nitric oxide (NO), which can cause both the inactivation of cytosolic aconitase and the stimulation of IRP1-IRE activity due to the disintegration of the Fe-S cluster[63, 64]. Similarly, a TLR-driven early metabolic shift in immune cells results in a rapid stimulation of glycolysis and subsequent oxidative phosphorylation failure attributable to NO-mediated damage to Fe-S proteins[65]. Moreover, TLR activation directly suppresses Fe-S cluster biogenesis by inhibiting the expression of many machinery components, which potentiates NO-mediated damage to Fe-S proteins[66]. Therefore, it leads to the transformation of macrophage energy metabolism into the glycolysis pathway, which is characteristic of M1 cells. It is worth noting that genes implicated in Fe-S cluster biogenesis, including ISCU and NSF1, are preferentially expressed in M2 macrophages that rely on mitochondrial oxidative phosphorylation for energy production[67].

Iron is also associated with amino acid catabolism. Tryptophan metabolism mediated by tryptophan monooxygenase helps maintain high iron level and effective mitochondrial respiration[68]. In addition, indoleamine 2,3-dioxygenase 1 (IDO1) can also breakdown tryptophan through the kynurenine pathway to form picolinic acid, a chelating agent of multiple elements in the human body, including zinc, iron, and chromium[69]. Picolinic acid can enhance interferon (IFN)-γ-induced NO production in macrophages[70], which may be due to iron deficiency caused by its ability to chelate iron and then induce the expression of NOS mRNA through HIF[71]. Iron chelation strongly induces the anti-inflammatory protein tristetraprolin, a downstream target of mTOR that binds to, and increases the degradation of TfR1 mRNA, providing a connection between inflammatory, metabolic, and iron-regulatory pathways[72, 73].
5.5 Macrophage ferroptosis

Ferroptosis is a newly discovered form of cell-regulated death defined by the accumulation of iron-dependent lipid hydroperoxide to a deadly level, which is morphologically, biochemically, and genetically distinct from apoptosis, necrosis, and autophagy characteristics. It is known that a few molecules are involved in the defense system against ferroptosis. Glutathione peroxidase 4 (GPX4), which catalyzes the conversion of lipid hydroperoxide to lipid alcohols, is the key protective enzyme against lipid peroxidation, and its function depends on its cofactor glutathione (GSH). The cystine/glutamate antiporter system Xc⁻ is required for GSH synthesis because it imports cystine in exchange for intracellular glutamate[74]. Loss of intracellular GSH results in ROS accumulation, and ROS leads to ferroptosis through the excessive oxidation of membrane lipids[74]. On the other hand, ferroptosis can be promoted by enhanced expression of acyl-CoA synthetase long-chain family member 4 (ACSL4)[75], which can enrich cellular membranes with long polyunsaturated fatty acids.

A recent study confirmed the role of ferroptosis in atherosclerosis. Ferroptosis inhibition can alleviate atherosclerosis by lowering lipid peroxidation and endothelial dysfunction in mouse aortic endothelial cells[5]. A lower GPX4 level has been linked to the development of atherosclerosis, while overexpression of GPX4 has been shown to reduce lipid peroxidation and ameliorate atherosclerotic lesions in Apoe⁻/⁻ mice[76]. In addition, the severity of atherosclerosis was positively correlated with ACSL4 expression, suggesting that ferroptosis is closely related to the occurrence and progression of atherosclerosis[77]. An inhibitor of ferroptosis, the free-radical-trapping antioxidant ferrostatin-1 alleviates iron overload and lipid peroxidation, reduces high lipid-induced ROS generation, effectively reduces ferroptosis, and alleviates atherosclerosis[78].

Macrophage ferroptosis has attracted much attention because of its important role in iron handling and lipid metabolism. However, data suggest that macrophages are not significantly susceptible to pro-stimulation[16]. In the microenvironment of advanced atherosclerotic plaques, macrophages can phagocytose large amounts of heme iron, but macrophages safely degrade heme by increasing their HO-1 expression. It has also been shown that, compared with M2 macrophages, M1 macrophages are insensitive to pharmacologically induced ferroptosis, which is dependent on NO production[79]. In addition, safe storage of iron by ferritin in M1
macrophages may confer higher resistance to ferroptosis. However, these homeostatic mechanisms can be overwhelmed, leading to cell death. Studies have confirmed that after rapid uptake of large numbers of red blood cells, increased HO-1 is not sufficient to prevent ferroptosis in macrophages[80]. Iron overload, which has no apparent effect on normal macrophages, also induces ferroptosis after exposure to oxLDL[81]. Further studies are needed to investigate whether macrophage ferroptosis exists and affects atherosclerosis progression.

6. Iron Metabolism in Macrophages as a Potential Therapeutic Target in Atherosclerosis

Several studies in experimental models have focused on how the changes in the hepcidin-FPN axis impacts the development of atherosclerosis. Deleting the hepcidin gene (Hamp) reduces macrophage iron in Hamp−/−Ldlr−/− mice, which is linked to a reduction in proinflammatory cytokines in macrophages and protection from atherosclerosis[57]. At the same time, the increase in serum iron treated with iron dextran in Hamp+/+Ldlr−/− mice failed to protect against atherosclerosis, which further confirmed that reducing macrophage iron rather than increasing serum iron has a protective effect. Other studies have also discovered a relationship between the hepcidin pathway and macrophage cholesterol metabolism. Dorsomorphin and its derivative, LDN 193189 (LDN), are small molecular inhibitors that reduce hepcidin expression and macrophage iron levels[82]. In Apoe−/− mice, LDN reduced foam cell production and delayed atherosclerosis progression by increasing cholesterol excretion from macrophages[83]. Therefore, low hepcidin causes enhanced FPN function, which can reduce macrophage iron, thereby alleviating atherosclerosis.

However, several atherosclerotic models of FPN mutant mice have shown conflicting results. In Apoe−/−FPNwt/C326S knock-in mice, a gain-of-function mutation of FPN causes a constitutive efflux of iron into the bloodstream due to a disturbing interaction between hepcidin and FPN[32]. Beyond the ability of transferrin to bind with iron, the formation of nontransferrin-binding free iron was supposed to be the reason for the aggravation of atherosclerosis. Moreover, iron chelation generates a protective effect on atherosclerosis[32]. Another mouse model, Apoe+/−ffe mutants, a heterozygous H32R mutation in FPN that causes iron accumulation in macrophages, fails to promote atherosclerosis[84]. To more precisely determine the direct in vivo effect of macrophage iron accumulation on the progression of atherosclerosis, we generated Apoe−/− mice with macrophage-specific
Fpn1 deficiency (Apoe\textsuperscript{-/-}Fpn1\textsuperscript{LysM/LysM})\textsuperscript{[33]}. Fpn1 deficiency in macrophages significantly accelerates the progression of atherosclerosis in mice by promoting the formation of foam cells. Furthermore, systemic iron chelation can alleviate atherosclerosis in Apoe\textsuperscript{-/-}Fpn1\textsuperscript{LysM/LysM} mice\textsuperscript{[33]}.

Not only does macrophage iron accumulation in the development of atherosclerosis show conflicting results\textsuperscript{[84]}, but a high-iron diet on atherogenesis has also been reported that 100 times as much more iron content than a standard iron diet was fed and that much body iron in serum and tissue could reduce atherosclerosis by 50\%\textsuperscript{[85]}. Then, we questioned whether the rapid iron flux in macrophages is the key, regardless of the elevated serum and tissue levels of iron. However, most studies support the modified iron hypothesis. Dietary iron loading exacerbates atherosclerosis\textsuperscript{[86]}. Reduced macrophage iron level, either through treatment with an iron chelator\textsuperscript{[87]} or an iron-deficient diet\textsuperscript{[88]}, reduced atherosclerosis, implying that decreased intracellular iron level protect against atherosclerosis.

Moreover, in a posthoc analysis of the Iron in Atherosclerosis Study (FeAST), ferritin reduction – not lipid changes – with statin therapy plus phlebotomy was associated with lower mortality, nonfatal myocardial infarction, and stroke in patients with established peripheral arterial disease\textsuperscript{[89]}, implying that the pleiotropic effects of statins may be mediated by iron and inflammation\textsuperscript{[90]}.

Notably, in CAD patients, a J-shaped relationship between systemic iron status and cardiovascular mortality was revealed\textsuperscript{[91]}. Iron deficiency has been shown to negatively affect individuals with coronary artery disease, heart failure, and pulmonary hypertension, as well as patients undergoing cardiac surgery\textsuperscript{[92]}. Furthermore, reducing macrophage iron levels in late-stage lesions (those with intraplaque hemorrhage) may accelerate plaque progression via VEGF-mediated increases in permeability, angiogenesis, and inflammatory cell recruitment\textsuperscript{[50, 93]}. Iron deficiency can also enhance atheroma inflammation through the p38 MAPK-NF-κB-EMMPRIN/MMP-9 pathway\textsuperscript{[94]}. Therefore, more studies are needed to determine the precise clinical consequences of altering iron homeostasis.

7. Conclusions and perspectives
The iron hypothesis has promoted many studies on the relationship between systemic iron and atherosclerosis. The refinement of the iron hypothesis has drawn more attention to macrophage iron. Macrophages play a critical role in iron homeostasis
and are crucial in atherosclerosis initiation, progression, and instability. Altered intracellular iron metabolism in macrophages is closely related to macrophage polarization, inflammatory factor production, lipid processing, angiogenesis, and ferroptosis, which affect the progression of atherosclerosis. Therefore, macrophage iron is a potential therapeutic target for atherosclerosis. However, more and more questions are being raised and need to be discussed and investigated.

Interestingly, hepcidin is a very important player in atherosclerosis, but its importance does not occur through the interaction between hepcidin and FPN. There are a few pieces of evidence to support it. First, hepcidin deficiency protects against atherosclerosis[57]; HFE C282Y homozygotes with hepcidin deficiency reduced low-density lipoprotein cholesterol in humans [95] and in mice as well[96], which translated into reduced atherosclerosis burden. Macrophage-knockout Fpn would cause severe anemia if we accepted that macrophages are crucial cells for recycling iron and that this iron is transported by FPN back to circulation. Mice with macrophage-knockout Fpn have mild anemia[17], suggesting another unknown exporter or another way to export iron, such as ferritin secretion, in macrophages. FPN is a key protein for iron intestinal absorption and embryo development because disrupted interaction between FPN and hepcidin triggers typical type IV hemochromatosis[97], and knockout of Fpn in mice is embryonic lethal[98]. In contrast, Apoe−/−Fpnwt/C326S knock-in mice exhibit not only iron overload but also exacerbated atherosclerosis, and iron chelation alleviates the development of atherosclerosis[32], implying that iron matters. The results may not be contradictory to the etiology that the incidence of atherosclerosis decreases in patients with hemochromatosis as life-long iron overload[9], likely because most hemochromatosis patients are mutations of HFE with a low hepcidin level, rather than Fpn mutation with high hepcidin level at late life (reviewed in [99]). Fpn mutation-triggered hemochromatosis is different from types I-III, which have a common primary hepcidin deficiency. FPN disease is prevalent in African individuals but very rare. Most likely, their atherosclerosis manifestation has not yet attracted attention in Africa or has not been counted statistically in the database of atherosclerosis risk in patients with haemochromatosis.

Another neglected phenomenon is cellular iron dynamics. It is commonly accepted that iron homeostasis is very important. However, we do not know how dynamic the homeostasis is. Is iron homeostasis maintained by slow iron ins and outs
or by active ins and outs? The systemic iron overload in any type of hemochromatosis would make the iron load in macrophages higher in patients with hemochromatosis than in healthy controls. However, high or low level of hepcidin makes the difference between pro-atherosclerosis and anti-atherosclerosis, suggesting that iron dynamics matter. The enhanced function of Fpn in \( Apoe^{-/-}Fpn^{wt/C326S} \) mice \[32\] and silenced function of Fpn in \( Apoe^{-/-}Fpn^{lysM/lysM} \) mice \[33\] generate similar symptoms of aggravated atherosclerosis. The possibility is that another iron exporter responds to increased hepcidin in \( Apoe^{-/-}Fpn^{wt/C326S} \) mice to block iron efflux, virtually a similar consequence to macrophage iron accumulation in \( Apoe^{-/-}Fpn^{lysM/lysM} \) mice. How these two iron exporters balance or how much each iron exporter contributes to the iron dynamics are interesting questions.

Here are the proposed new messages: (i) Macrophage iron status and dynamics matter in atherosclerosis. (ii) Fpn plays a major role in iron absorption from the diet and embryo development but a relatively minor role in macrophage iron recycling. (iii) There is, very likely, an unknown iron exporter in macrophages that responds to hepcidin, whose low level facilitates iron export and antiatherosclerosis. (iv) How iron affects macrophage phenotypes depends on the context of the macrophage microenvironment. (v) Hepcidin downregulation or blockage is a therapeutic direction to intensify iron export from macrophages. (vi) Macrophage ferroptosis in atherosclerotic plaques needs further confirmation, and its regulation and intervention need to be further investigated.

**Funding**

This work was supported by the National Natural Science Foundation of China (Nos. 81870348 and 31871201)

**Conflict of Interest**

These authors declare that they have no conflict of interest.

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Figure Legends

Figure 1. Regulation of systemic iron metabolism
Organs and cell types involved in systemic iron homeostasis are shown. Dietary iron absorption: Duodenal enterocytes absorb dietary iron via divalent metal transporter 1 (DMT1) located on the apical surface upon reduction of Fe³⁺ to Fe²⁺ by Fe-reductase (DcytB). At the basolateral membrane, ferroportin (FPN) cooperates with Feoxidase (hephaestin) to convert Fe²⁺ to Fe³⁺. Iron utilization: Plasma transferrin (Tf) captures and circulates iron in the body. By binding to transferrin receptor 1 (TfR1) and subsequent endocytosis, iron-loaded transferrin (Holo-Tf) provides iron to all types of cells. Iron recycling: Iron is recycled by macrophages from senescent red blood cells. The uptake of Hb-Hp and
Hx-heme is mediated by CD163 and LDL (low-density lipoprotein)-related receptor 1 (LRP1, also known as CD91). Heme oxygenase 1 (HO-1) catabolizes intracellular heme-Fe for inclusion into the cytosolic labile iron pool or cellular ferritin pool or trafficked into the mitochondria. Non-Tf-bound iron (NTBI) is imported via DMT1. Ceruloplasmin (Cp) facilitates iron export by FPN by oxidizing Fe^{2+} to Fe^{3+}, allowing apo-Tf to sequester it. Iron regulation: The hepatic hormone hepcidin modulates the stability of FPN, which controls iron outflow from these cells.

**Figure 2. Macrophage subpopulations and iron states in atherosclerotic plaques**

Interferon (IFN) and lipopolysaccharide (LPS), as well as oxidized low-density lipoprotein (oxLDL) and cholesterol crystals, are stimuli for M1 polarization; IL-4 is an inducer of M2 polarization, oxidized phospholipids (oxPL) for the Mox phenotype, and the Hb/Hp complex for the M(Hb) phenotype. M1 macrophages display proinflammatory and iron retention profiles. Iron activates the Toll-like receptor (TLR)/nuclear factor kappa-light-chain-enhancer of activated B cells (NFkB) signaling pathway, which is in charge of macrophage inflammatory activity. OxLDL and iron stimulate the TLR4 pathway, resulting in the autocrine production of hepcidin. This results in the exacerbation of iron accumulation, ROS production, and the inflammatory response through a positive feedback loop. Furthermore, iron retention promotes foam cell formation by increasing CD36-mediated cholesterol absorption and decreasing ABC transporter ABCA1/ABCG1-mediated reverse cholesterol efflux via interference with CYP27A1 and liver X receptor (LXR) signaling, respectively. M (Hb) and M2 macrophages play a crucial role in iron handling and prevent foam cell formation. While these nonfoam M(Hb) macrophages are supposed to be antiatherogenic in theory, intracellular iron deficiency causes hypoxia-inducible factor 1α (HIF1α) stability and vascular endothelial growth factor (VEGF) release, which has proatherosclerotic effects by increasing vascular permeabilization and intraplaque neoangiogenesis. Mox macrophages exhibit reduced phagocytic capacity and express antioxidant genes. However, iron retention and lipid accumulation in Mox macrophages likely contribute to lesion development and plaque instability in atherosclerosis.
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Lipid deposit
ABCA1
ABCG1
CD36
NF-κB
TLR4
IL-6
IL-1β
TNF-α
IL-10
ARG-1
CD206
CD163
HIF-1α
HO-1
Nrf2
Hepcidin
oxPL
oxLDL/Cholesterol
Fe2+
Fe3+
VEGF
CObiliverdin

Pro-inflammatory
Oxidative stress
Foam cell formation

Anti-inflammatory
Reduced oxidative stress
Prevent foam cell formation

Anti-inflammatory
Reduced oxidative stress
Prevent foam cell formation

Anti-oxidant properties
Low phagocytic capacities

Iron retention
Iron retention
Iron depletion
Iron depletion

Pro-inflammatory
Vegeterine
Intra-plaque neoangiogenesis; vascular permeability

Iron status
Effect on atherosclerosis
Highlight

Macrophages with the M1, M2, M(Hb), Mox, and other phenotypes have been defined with different iron-handling capabilities related to the immune function and immunometabolism of macrophages, which influence the progression of atherosclerosis. Altered intracellular iron metabolism in macrophages is closely related to macrophage polarization, inflammatory factor production, lipid processing, angiogenesis, and ferroptosis, which affect the progression of atherosclerosis. Recent advances in the field include:

- Macrophage iron status and dynamics matter in atherosclerosis.
- Cellular iron modulates macrophage polarization.
- Macrophage iron is a potential therapeutic target for atherosclerosis.
Lipid deposit
Pro-inflammatory

Anti-inflammatory

Hepcidin
oxPL
Fe2+
Fe3+

Angiogenesis
M1 M2 M(Hb) Mox
IFNγ, LPS, oxLDL, cholesterol
IL-4
Hb-Hp
oxPL
ROS
Hb-Hp
oxPL
FPN
Cp

Pro-inflammatory
Lipid deposit

Anti-inflammatory

Angiogenesis

Pro-inflammatory
Lipid deposit

Pro-inflammatory
Lipid deposit

Hepcidin
oxPL
FPN
Cp