IRON BIOLOGY

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ABSTRACT

Iron is a fundamental element for biological life, starting from bacteria till humans. Iron is essential for cell function and survival, energy production and metabolism, whereas increased levels cause oxidative stress. It is also a constituent of haemoglobin and thus it is necessary for oxygen transportation through the body. Given these multiple functions, the regulation of iron metabolism is complex and tight coupled with oxygen homeostasis at tissue and cellular levels, thanks to the interaction with the hypoxia inducible factor (HIF) system.

In patients with chronic kidney disease (CKD), iron deficiency significantly contributes to anaemia development. This frequently overlaps with chronic inflammation, causing iron-restricted erythropoiesis. To add further complexity, metabolic hyperferritinemia may, on one side, increase the risk for CKD and, on the other, overlaps with functional iron deficiency. Excessive intracellular iron in certain cell types during CKD can also mediate cellular death (called ferroptosis), and contribute to the pathogenesis of kidney damage, atherosclerosis and vascular calcifications.

This review is aimed at broadening the perspective of iron metabolism in the setting of CKD not just as a contributor to anaemia in CKD patients, but also as an important player with an impact on cell metabolism, renal fibrosis, and the cardiovascular system.

Keywords: anaemia, cardiovascular disease, chronic kidney disease, ferroptosis, inflammation, iron, metabolic hyperferritinaemia, vascular calcification

INTRODUCTION

Over the years, the management of iron status has been a controversial issue in the management of patients with chronic kidney disease (CKD). In the pre-erythropoiesis-stimulating agents (ESA) era, when correction of severe anaemia relied on regular transfusions, the problem was iron accumulation with the risk of haemosiderosis. With the advent of ESAs, we moved from the removal of potentially toxic iron to its administration to support the production of haemoglobin (Hb) in new red blood cells.

In more recent years, we have had to deal with inflammation and the consequent emergence of functional iron deficiency, where iron stores are high but little or nothing is available to the erythron, due to elevated hepcidin levels that inhibit iron mobilisation, and thereby rendering less effective further administration of iron to correct the anaemia.

Given the cost of ESA therapy and studies suggesting an increase in cardiovascular mortality with high doses of ESAs, we have moved from the physiological use of iron to compensate for losses, to a pharmacological use to reduce ESA doses and to supply iron to support new Hb synthesis thereby preventing depletion of iron stores.

The advent of hypoxia-inducible factors-prolyl hydroxylase inhibitors (HIF-PHIs) has further modified iron therapy, due to possible increased mobilisation of iron from stores and improved intestinal absorption.

In this review, we aim to broaden the perspective and focus not only on the traditional view of iron as a contributor to anaemia in CKD patients, but also as an important player with an impact on cell metabolism, renal fibrosis and the cardiovascular system.

INFLAMMATION AND IRON-RESTRICTED ERYTHROPOIESIS

Besides classical erythropoietin (EPO) deficiency, systemic inflammation is increasingly recognised as a critical contributor to the pathophysiology of anaemia in CKD. Accordingly, this condition is nowadays often included in the broad category of “anaemia of

inflammation" [1]. The mechanisms underlying low-grade chronic inflammation in CKD are heterogenous. They include cell injury by oxidative stress and the uraemic milieu with release of damage associated molecular patterns (DAMPs) and activation of the inflammasomes [2], decreased clearance of C-reactive protein (CRP), production of interleukin-6 (IL-6) and other pro-inflammatory cytokines like interleukin 1-β (IL1-β) and tumour necrosis factor α (TNF-α), increased incidence of infections with release of pathogen-associated molecular patterns (PAMPs), the widespread presence of atherosclerosis and vascular damage [3].

Pro-inflammatory cytokines inhibit erythropoiesis either directly or indirectly. Both IL1-β and TNF-α suppress EPO production. In addition, TNF-α also reduces EPO activity by blunting the response of erythroid progenitors [4]. On the other hand, inflammation drives profound changes in iron metabolism, leading to the upregulation of hepcidin and ferritin, with the downregulation of transferrin. Indeed, the binding of IL-6 to its membrane receptor (IL-6R) activates the janus kinase (JAK) signal transducer and activator of transcription (STAT) signalling, eventually enhancing transcription of the hepcidin gene (HAMP) [5]. This integral part of the innate immune response ultimately leads to iron sequestration into macrophages, with the scope of limiting the availability of the micronutrient essential for most of the invading pathogens, but inevitably resulting in iron-restricted erythropoiesis in the host. In CKD, such "functional iron deficiency" can be related to increased transcription of hepcidin by inflammation and decreased renal clearance of this small peptide hormone [6,7]. Iron homeostasis in CKD is further complicated by the fact that numerous factors can lead to absolute iron deficiency, namely malnutrition and blood losses (for example, through haemodialysis, repeated blood sampling, gastrointestinal bleeding favoured by antithrombotic therapies or platelet dysfunction), especially in advanced disease. This overlap between functional and absolute iron deficiency makes the interpretation of

laboratory studies of iron metabolism exceedingly difficult in CKD or heart failure (HF) patients, with uncertain cut-offs to aim at during iron therapy.

METABOLIC HYPERFERRITINAEMIA

Apart from being an inflammatory marker, hyperferritinaemia is often associated with metabolic dysfunction in the general population, defined by the presence of overweight or type 2 diabetes or multiple metabolic features of insulin resistance, including hypertension, atherogenic dyslipidaemia (low HDL cholesterol and high triglycerides) and steatotic liver disease (SLD) [8]. This condition has recently been defined metabolic hyperferritinaemia (MHF) [9]. MHF reflects alterations in iron metabolism related to lipotoxicity and subclinical inflammation that may facilitate body iron accumulation in predisposed individuals, and is associated with more severe insulin resistance, metabolic alterations and liver damage, than in patients with metabolic alterations with normal ferritin levels, resulting in an increased risk of cardiovascular, hepatic disease, and cancer.

Genetic variants and iron tissue levels contribute to modulating ferritin serum levels in individuals with metabolic dysfunction [10]. Conversely, iron depletion may improve insulin resistance and liver damage, raising the hypothesis that iron accumulation might be implicated in the pathogenesis of insulin resistance and organ damage [8].

MHF is frequently observed in individuals with CKD as metabolic dysfunction represents the main cause or kidney disease in the population, in particular in men with obesity, type 2 diabetes and arterial hypertension. Notably, in patients with type 2 diabetes, hyperferritinaemia and altered iron metabolism is a risk factor for glomerular filtration rate (GFR) decline over time [11] and acute kidney injury (AKI) [12]. Indeed, the presence and severity of SLD, which correlates with ferritin levels, is considered among risk factor for CKD development [13]. However, due to the development of inflammation with the deterioration of kidney function, the overlap of MHF with functional iron deficiency despite

adequate iron stores complicates the decision of whether to administer iron therapy in anaemic CKD patients.

FERROPTOSIS IN THE KIDNEY, HEART AND ARTERIES

Ferroptosis indicates an iron-dependent distinct form of non-apoptoptic cell death initiated by intracellular oxidative stress [14]. It was originally described following studies on small molecules (e.g. elastin) able to inhibit the uptake of cystine, with ensuing reduced activity of the most important constitutive antioxidant, glutathione peroxidase (GSH-Px4), eventually leading to lethal lipid peroxidation. The key players in ferroptosis are the intracellular levels of the labile iron pool and antioxidants. Accordingly, ferroptosis can be inhibited by iron chelators (e.g. deferoxamine) and other synthetic compounds like ferrostatin-1.

AKI and CKD are typically associated with increased oxidative stress; unsurprisingly, ferroptosis is increasingly recognised as an important player in their pathophysiology [15]. It may also represent a significant contributor to the pathogenesis of several types of cardiovascular disease, including atherosclerosis, vascular kidney damage (AKI), and diabetic nephropathy. Ferroptosis can be pharmacologically modulated through several compounds, most of them experimental. An anti-ferroptosis activity has been described for some established drugs, including the HIF-PHI roxadustat [16]. Further research is needed to determine whether targeting ferroptosis may be a valid therapeutic option for renal diseases.

IRON AND KIDNEY FIBROSIS

Macrophages play an important role in kidney fibrosis and at the same time, are critical for the maintenance of systemic iron homeostasis [5,17]. In CKD, systemic iron deficiency is associated with intracellular labile iron pool (LIP) deficiency in kidney macrophages and

elevation of the transferrin receptor 1 (TfR1) as an attempt to acquire more iron. LIP-deficient macrophages present defective antioxidant response, accumulation of reactive oxygen species (ROS) and increased production of pro-inflammatory cytokines and pro-fibrotic factors (e.g. TGF-β), suggesting a role for macrophage iron deficiency in cell pro-inflammatory and pro-fibrotic skewing in CKD [18].

CKD is a highly pro-oxidative condition and oxidative stress has been implicated in kidney fibrosis and disease progression [19]. Macrophage iron deficiency likely aggravates cell oxidative stress in this condition due to the inappropriate expression and activity of enzymes of the anti-oxidant response which are iron-dependent (e.g. catalase, peroxidases, heme-oxygenases). Sterile inflammation and fibrosis development are likely promoted by the activation of kidney macrophages through these mechanisms [18].

Interestingly, the correction of macrophage iron status achieved through iron infusion or genetic LIP repletion reduces oxidative stress, decreases the production of pro-inflammatory cytokines, and mitigates fibrosis in CKD animal models, suggesting that iron therapy protects against kidney fibrosis by suppressing macrophage inflammatory and fibrotic skewing [18]. Finally, iron significantly suppresses TGF-β-driven fibrotic response of macrophages, further inhibiting pro-fibrotic mechanisms in CKD [18]. Thus, targeting intracellular iron deficiency of kidney macrophages in CKD can be exploited as therapeutic opportunity to mitigate disease progression.

IRON AND ATHEROSCLEROSIS

Although iron has long been suspected to promote the development of atherosclerosis due to its role in the generation of ROS, whether iron is involved in the development of atherosclerosis has remained a controversial and unresolved issue [20].

The fact that patients with haemochromatosis do not show an increased incidence of atherosclerosis is often cited as the most convincing evidence against a detrimental role of iron in atherosclerosis [21].

ApoE-deficient mice have been widely used in preclinical atherosclerosis studies because of their propensity to spontaneously develop atherosclerotic lesions with features similar to those observed in humans [22,23]. The increased iron contents have been detected in advanced lesions from ApoE-deficient mice in which the staining was present in intima enriched in foam cells as well as in smooth muscle cells of the media layer. By Oil Red O staining, RT-PCT and western blot analysis, DAB-enhanced Perl's staining and immunohistochemistry, the existence of atherosclerosis was confirmed with a significant increase in iron content, and expression of ferritin light chain (FTL) and ferritin heavy chain (FTH) mRNAs and proteins in the aortic tissues of ApoE-deficient mice aged 28-weeks-old [24] (Figure 1). The increased iron deposition in the aortic tissues in ApoE-deficient mice has been observed been confirmed in multiple preclinical studies [25,26] and has found support in some clinical studies [26,27]. The increased cell-iron uptake by the IRP/TfR1 pathway is probably one of the reasons for iron accumulation in aortic tissues in ApoE-deficient mice. Several recent preclinical studies indeed supported a causal link between iron and atherosclerosis using mouse models of iron overload, achieved either via genetic mutation, via iron-enriched diet or iron infusion [22,23,24,25,26,27]. Since the majority of the investigations have been performed on mouse models with elevated LDL, iron can be considered most likely as a modifier of atherosclerosis in association with an altered lipid profile.

NON-TRANSFERRIN-BOUND IRON AND VASCULAR DAMAGE

CKD patients have an accelerated atherosclerosis, increased risk of thrombotic-ischemic complications, and excessive mortality rates when compared to the general population.

While on the one hand a clear association exists between systemic iron deficiency, which hallmarks CKD, and cardiovascular diseases, also iron excess has been associated with CVD development [23,27]. Anaemia and limited iron availability likely impair cardiac function, whereas iron excess causes vascular damage through the formation of non-transferrin-bound iron (NTBI). NTBI contributes to endothelial dysfunction by inducing the pro-inflammatory activation of the vascular endothelium and affecting the functionality and survival of endothelial and vascular smooth muscle cells (ECs; VSMCs). Exposure of ECs to NTBI triggers increased expression of adhesion molecules (e.g. VCAM1, ICAM1, E-selectin, P-selectin), secretion of chemoattractants (monocyte chemoattractant protein MCP-1), intracellular ROS formation and cell death [28]. This might result in vaso-permeabilisation and increased monocyte adhesion which likely promote the infiltration of low-density lipoproteins (LDL) into the subendothelial space and recruitment of monocytes, which eventually turn into foam cells. In addition, iron impairs endothelium-dependent vasorelaxation and triggers arterial stiffness by inducing ROS formation and decreasing nitric oxide (NO) bioavailability through suppressed endothelial NO synthase (eNOS) expression/activation and/or enhanced NO oxidative consumption [28]. NTBI has also the ability to increase the expression of vascular endothelial growth factor (VEGF) in ECs and VSMCs. Elevated VEGF has been implicated in atherosclerosis due to its pro-inflammatory and permeabilising action on the vascular endothelium [27,28]. Similarly, when exposed to NTBI, VSMCs develop iron overload, produce ROS and undergo apoptosis associated to the release of MCP-1, further stimulating the recruitment of monocyte, and promoting inflammation and eventually plaque progression [28]. These observations point at a clear involvement of both iron deficiency and overload in cardiovascular complications, suggesting that the maintenance of iron balance and transferrin saturation within proper limits is critical for cardiovascular health [27].

IV iron administration could be an additional factor exposing patients to increased labile iron, oxidative stress, and endothelial dysfunction. The effect is more likely to occur in patients with high hepcidin levels blocking iron uptake. It could also depend on the type of intravenous iron formulation, as large molecular-weight molecules theoretically release less free iron into the circulation [29]. However, IV iron could be protecting, by improving mitochondrial function and upregulating anti-oxidant pathways [30].

IRON AND VASCULAR CALCIFICATION

In CKD, an imbalance in iron homeostasis can have detrimental effects on the body, including its association with vascular calcifications (Figure 2). Vascular calcification is a pathological process characterised by the accumulation of calcium salts in the walls of blood vessels with vascular smooth muscular cells undergoing a phenotypic switch to osteoblast-like cells. This process shares similarities with bone mineralisation, as it involves the deposition of hydroxyapatite crystals within the vascular tissues. Whereas some degree of calcification can occur as a part of the aging process, excessive and premature calcification of blood vessels is linked to atherosclerosis, coronary artery disease, and other cardiovascular complications [31].

The influence of iron on vascular calcification is a complex and evolving topic within cardiovascular research. Whereas iron is essential for various physiological functions, including oxygen transport and cellular metabolism, its effects on vascular calcification can vary based on factors such as the form of iron, its concentration, and the specific conditions of the individual [32]. There is some evidence that suggest that iron might have a protective effect against vascular calcification in specific circumstances. Iron deficiency, for example, has been linked to impaired collagen synthesis and tissue integrity, which could potentially contribute to calcification. In these cases, addressing iron deficiency through appropriate supplementation might improve tissue health and potentially reduce

the risk of calcifications. Moreover, in *in vitro* studies, iron citrate is able to prevent and partially revert the calcium– phosphate crystal deposition in the classical model of vascular smooth muscle cells calcification [33,34].

Excessive elevation in iron levels, particularly in the form of NTBI, can promote oxidative stress and inflammation within vascular tissues. These processes contribute to the activation of VSMCs and endothelial dysfunction, creating an environment conducive to calcium deposition. Furthermore, iron has been found to influence various factors implicated in calcification regulation, including matrix Gla protein (MGP) and fetuin-A. These proteins are involved in inhibiting the precipitation of calcium salts and maintaining mineral balance in tissues. Dysregulation of these calcification inhibitors due to iron-related oxidative stress can disrupt their protective functions, leading to accelerated vascular calcification [35,36].

It is worth noting that research in this field is ongoing, and conclusions can vary based on experimental conditions, study populations, and methodologies. The effects of iron on vascular calcification likely depend on a delicate balance between its essential functions and its potential role in oxidative stress and inflammation.

Continued research into the molecular mechanisms connecting iron metabolism and vascular calcification could pave the way for innovative treatments aimed at preventing or slowing down the progression of cardiovascular diseases.

IRON METABOLISM AND ITS CONNECTION WITH THE HIF SYSTEM

The control of iron metabolism is complex, with several pathways involved. From the nephrological perspective, the HIF-PHD axis is the most important, displaying connections with other cellular iron regulatory pathways, such as iron regulatory proteins (IRP1 and IRP2) and the iron-responsive element (IRE) signalling [37].

HIF activation is known to decrease serum hepcidin; the molecular mechanisms contributing directly or indirectly to this effect are still debated. Increased erythropoiesis indirectly suppresses hepcidin synthesis [38]; erythroferrone is the mediator of the process. Said that, HIF-1α can bind the hepcidin promoter and suppress hepcidin gene transcription directly [39]. This has been questioned since the decrease in hepcidin mRNA was not reversed by HIF-1α or HIF-2α knock-down or by depletion of the HIF and IRP target TfR1 [40]. HIF1α and HIF-2α can also upregulate the type II transmembrane serine proteinase (TMPRSS6); this enzyme antagonises hepcidin induction by bone morphogenetic proteins (BMPs) in the liver [41].

Hepcidin controls ferroportin activity downstream by directly binding within its central cavity and stopping the transportation of iron from the cytoplasm to the extracellular space or by inducing endocytosis and proteolysis of ferroportin [42]. This molecule is the iron exporter in the basolateral membranes of duodenal enterocytes, in iron-recycling macrophages, and in iron-storing hepatocytes [43]. Other steps of iron metabolism, such as the transferrin receptor, are directly regulated by HIF-1α [44].

HIF-2α activation enhances iron absorption directly from the gut. This happens within a uniquely steep oxygen gradient from the richly perfused gastrointestinal mucosa to the anaerobic lumen [45]. In conditions of iron deficiency or hypoxia, HIF-2α activates the transcription of the divalent metal transporter 1 (DMT1) and the ferric reductase DcytB at the luminal side of enterocytes and that of ferroportin at the basolateral one [46].

The HIF system also controls iron utilisation for Hb synthesis. Indeed, it enhances the activity of ferrochelatase, the enzyme that catalyses the insertion of iron into protoporphyrin to form haeme [47].

On the other hand, iron is a counter-regulator of HIF activity since PHD enzymes are Fe(II)/2-oxoglutarate-dependent oxygenases (the lower iron content, the lower PHD

activity and the longer HIF-1α half-life). This also happens locally, as it has been demonstrated in the gut. Interestingly, ascorbic acid, known to increase intestinal iron absorption, also stimulates PHD activity [48], contributing to tuning down the HIF system in the presence of adequate iron stores.

EFFECTS OF ERYTHROPOIESIS STIMULATING AGENTS AND HIF-PH INHIBITORS ON IRON METABOLISM

CKD patients are frequently anaemic because of insufficient EPO but also because of iron deficiency. Anaemia is associated with worsening left ventricular hypertrophy, dyspnoea, thrombotic events and reduced quality of life (QoL) [49,50]. These observations inspired studies to increase Hb levels with ESAs. While some measures of heart function in subjects targeted to higher Hb did better, cardiovascular endpoints and survival were the same or worse with increased levels of strokes and thrombotic events in some trials and there was little/no improvement in QoL [51].

There was a negative association between targeting a higher Hb and hyporesponse [52,53] but subjects that achieved the target Hb did better and there was improvement in fatigue [54]. Since a cause of hyporesponse is iron deficiency, parenteral iron administration was considered. In a retrospective analysis, intravenous (IV) iron at moderate doses improved cardiovascular and all-cause mortality in haemodialysis patients [55].

Another hypothesis was that HIF-PHIs, which has been reported to improve Hb responses and also increase mobilisation of iron by HIF-PHIs, may provide better outcomes. However, results of phase 3 trials did not support this and in some studies subjects randomised to HIF-PHI had increases in major adverse cardiac events (MACEs) and relative increases in thrombotic events [49].

Anaemia correction whether by ESAs or HIF-PHIs results in mobilisation of iron to support Hb synthesis. This is testified by the fact that increased erythropoiesis due to ESA therapy is followed by a reduction in serum hepcidin. To increase Hb by 1 g/dL requires 170 mg of iron. Given that iron stores in healthy individuals are generally comprised between 1 and 3 g, anaemia correction of 2 g/dL (typical of anaemia correction studies) and enhanced erythropoiesis whether driven by ESAs or HIF-PHIs can deplete iron stores by up to 1/3, thereby reducing considerably iron needed for other essential processes. Multiple studies support the possibility that iron depletion may explain the failure to see improvement with anaemia correction. Markers of iron-restricted erythropoiesis correlated with worse outcomes. Increased red distribution width (RDW) was associated with increased plaque rupture in patients with acute coronary syndromes [56] and increased risk of cardiovascular events [57]. Low hepcidin and low ferritin (measures of iron stores) were associated with increased three-year mortality among patients with HF [58], and outcomes were worse in patients with both low iron stores and anaemia than low hepcidin or anaemia alone. This suggests that iron depletion may explain the failure to improve outcomes in anaemia correction studies and that concomitant parenteral iron administration may provide health benefits.

Today, there is consensus that iron stores should be replete at the start and during treatment with either ESAs [59] or HIF-PHIs [60]. However, the optimal values of TSAT or serum ferritin for guiding the therapeutic choices in CKD patients are less clear. From one side, the data of the PIVOTAL trial have clearly shown that in incident haemodialysis patients with little inflammation, IV iron improves outcomes and reduces ESA doses and transfusion needs when administered in a proactive fashion (400 mg monthly, unless the ferritin concentration was >700 ng/mL or the transferrin saturation was ≥40%) [61]. However, the “reactive” control group had iron deficiency on average. Therefore, it is unclear whether the proactive approach up to ferritin 700 ng/mL is superior to an

intermediate correction of iron deficiency (serumferritin above 200 ng/mL for dialysis patients). Notably, this treatment strategy did not increase the infection risk (one of the feared complications of IV iron) [62]. Adding further complexity, observational studies show a U-shaped mortality curve following IV iron administration, with significant benefit at up to 400 mg/month of IV iron [63].

In ND-CKD patients, the FIND study showed that targeting serum ferritin between 400-600 ng/mL reduced the need for start ESA therapy or receive blood transfusions [64]. However, the trial was not powered to prove efficacy on hard endpoints. Functional iron deficiency is still a shadow in the knowledge since there is no strong evidence either showing benefits on outcomes or harms when treating patients with serum ferritin exceeding 500 ng/ml and low TSAT values [59].

IRON AND THE HEART

There is a bidirectional link between HF and CKD, whereby kidney dysfunction is common and associated with adverse outcomes in patients with HF [65], whilst HF is also a risk factor for future kidney failure [66]. Reduced kidney function is a risk factor for anaemia in people with HF and consequently anaemia has been reported to be associated with poorer quality of life in patients with HF [67]. However, correction of anaemia with ESAs did not show benefit in the Reduction of Events by Darbepoetin Alfa in HF (RED-HF) double-blind placebo-randomised controlled trial of darbepoetin in 2,278 patients with systolic HF and mild to moderate anaemia. There was no benefit of anaemia correction with darbepoetin on the primary end point of all-cause mortality or HF hospitalisation despite clear separation in the Hb between the groups [68]. Furthermore, there was an increased risk of stroke in the subgroup of patients with HF and CKD in the darbepoetin group [69].

Cellular studies highlight mechanisms by which iron may impact cardiomyocyte function giving insights into the mechanisms by which iron depletion due to anaemia correction with

ESAs may exacerbate HF, especially in the absence of administered parenteral iron. As heme, iron is essential for the synthesis of cytochromes, cellular iron deficiency has been demonstrated to decrease mitochondrial function in cardiomyocytes and consequently impair contractile function [70]. Myocardial tissue from the explanted failing hearts of patients undergoing cardiac transplantation has been shown to exhibit cellular iron depletion, increased oxidative stress and mitochondrial dysfunction [71].

Initial randomised controlled clinical trials have shown benefits with IV (but not oral [72]) iron on both exercise capacity and QoL in patients with HF [73,74]. More recently, the EFFECT-HF study (Effect of Ferric Carboxymaltose on Exercise Capacity in Patients with Chronic Heart Failure and Iron Deficiency), showed that intravenous ferric carboxymalthose significantly improved the peak oxygen volume, the NYHA, and patient global assessment in 174 patients with reduced EF [75]. Another small RCT was able to demonstrate only modest numerical improvement in parameters of quality of life [76]. Three large randomised controlled trials have recently reported which help define the role of IV iron in the management of patients with HF and iron deficiency. The Study to Compare Ferric Carboxymaltose With Placebo in Patients With Acute Heart Failure and Iron Deficiency (AFFIRM-AHF) compared ferric carboxymaltose to placebo in patients who have been stabilised following an episode of acute HF, and whilst there was no difference in cardiovascular death between the two groups, there was a significantly lower risk of subsequent HF hospitalisation with ferric carboxymaltose (RR 0·74; 95% CI 0·58–0·94, p=0·013) [77]. In the prospective, open label blinded end point Intravenous Iron Treatment in Patients With Heart Failure and Iron Deficiency (IRONMAN) trial in 1137 patients with chronic HF, allocated 1:1 to IV ferric derisomaltose or no treatment, ferric derisomaltose was associated with a lower risk of hospital admissions for HF and cardiovascular death compared to no treatment, albeit in prespecified analyses which were censored to take

account of the COVID19 pandemic [78]. By contrast, in the Ferric Carboxymaltose in HF With Iron Deficiency (HEART-FID) trial, which randomised 3065 people with HF and left ventricular ejection fraction <40% to either ferric carboxymaltose or placebo, there was no difference in the hierarchical composite outcome of death, hospitalisations for HF, or 6-minute walk distance [79].

In haemodialysis patients, the PIVOTAL trial discussed earlier demonstrated that proactive iron strategy was associated with both reduced risk of not only the primary end point (a composite of non-fatal myocardial infarction, nonfatal stroke, hospitalization for heart failure, or death), but also heart failure, hospitalisation, and myocardial infarction [80,81].

In summary, evidence from preclinical experiments shows that myocardial iron deficiency exacerbates cardiac dysfunction. Clinical trials demonstrate that IV iron is generally beneficial in people with HF, although the results demonstrate some inconsistencies across agents used and populations studied. Recently, the European Society of Cardiology upgraded its recommendations based on more recent evidence [82]. It suggests IV iron in patients with HF with reduced or mildly reduced ejection fraction and iron deficiency (defined on the basis of the inclusion criteria of the IRONMAN trial, i.e. a TSAT <20%) or serum ferritin <100 µg/L) to improve symptoms and quality of life and reduce the risk of HF hospitalization [82]. Notably, these recommendations differ from the indications for the CKD population in terms of aim of the treatment and iron parameters when to start or interrupt the therapy, because HF patients do not reach the same very high serum ferritin observed in inflamed CKD patients, especially if receiving haemodialysis. However, TSAT is a poor measure of iron availability because it is affected by the degree of stimulation of erythropoiesis. Surprisingly, it increases rather than decreases as a result of serum iron being mobilised to support erythropoiesis. In contrast,

ferritin and hepcidin behave in the opposite way, but may be confounded by inflammation. Better markers are mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC) and red cell distribution width (RDW). Notably, elevated RDW levels are associated with adverse outcomes in HF patients [83].

NOVEL POTENTIAL THERAPEUTICS TO MODIFY IRON METABOLISM AND ERYTHROPOIESIS

The discovery of the HIF system and the clinical development of agents modifying its activity have represented a significant achievement. However, apart from their anti-anaemic effect, HIF-PH inhibitors have not been capable of significantly improving patient outcomes. For this reason, CKD patients are still in need of new treatments to improve their underlying anaemia. Unfortunately, at present, little research is going on in the field for the development of new agents stimulating erythropoiesis (Table 1).

Peginasatide, an EPO mimetic stimulating the EPO receptor, was withdrawn from the US market following severe infusion reactions. A new version of the drug, pegmolesatide (Hansoh Pharmaceutical Group Co, Ltd, Shanghai, China) is now under clinical development for the treatment of non-dialysis (NCT03903809) and dialysis patients [84]. Apart from that, no other agents are in the pipeline for CKD.

Luspatercept has recently entered the market for the treatment of ineffective erythropoiesis in patients with beta thalassemia or myelodysplastic syndromes. Unfortunately, the clinical development in CKD of this drug and its analogue sotatercept was halted some years ago.

Recently, the possibility of increasing the number of erythrocyte bone marrow precursors has been suggested in a rat model of ESA hyporesponse [85].

Given that CKD patients often have a chronic inflammatory state and high hepcidin levels, reducing hepcidin levels could be valuable in ESA hyporesponse or to prevent iron

overload in the case of functional iron deficiency. Hepcidin levels could be decreased by inhibiting its synthesis or by blocking its function (Table 1).

NOX-H94, a Spiegelmer of the molecule, binds to human hepcidin with high affinity and blocks its biological function. Some years ago, the drug was shown to inhibit hepcidin-induced ferroportin degradation in cynomolgus monkeys with chronic inflammation and anaemia [86]. It was tested in a phase 2 study of hemodialysis patients hyporesponsive to ESA (NCT02079896). Its clinical development was then halted.

Another hepcidin antagonist is PRS-080, an anticalin antibody targeting hepcidin with high affinity and selectivity. A phase 2a study had been completed in haemodialysis patients with anemia (NCT03325621), but no further clinical development is foreseen. Anti hepcidin antibodies were also developed, but are still in the preclinical phase [87].

Bone morphogenetic protein (BMP)-6, together with BMP2 critically control hepcidin transcription via the BMP/SMAD pathway. A fully human anti-BMP6 antibody (KY1070, Kymab) has been developed and tested in animal models [88]; the drug is still in the preclinical phase. Another anti/BMP6 antibody (CSJ137, Novartis) was tested in a phase 2 clinical trial of anaemic haemodialysis patients (NCT02570854); the study is complete, but the results have not been published yet. Repulsive guidance molecule c/hemojuvelin (RGMc/HJV) is a co-receptor of BMP and contributes to the stimulation of hepcidin expression; two humanised anti-RGMc/HJV monoclonal antibodies were designed and tested in preclinical studies [89]. Small molecules targeting the type 1 ALK2 receptor are also of interest (BMP6 is a ligand of the ALK2 receptor) [90].

Momelotinib is a JAK1/2 and activin receptor type 1 (ACVR1) inhibitor (also known as ALK2); the pathway is enhanced by inflammation and upregulates hepcidn production. The drug has shown promising results in patients with myelofibrosis who were transfusion dependent [91].

Among is properties, heparin is known to inhibit hepcidin production; low anticoagulant heparins were developed targeting hepcidin; in preclinical studies they showed correction of anemia of chronic inflammation [92]. Sevuparin is a heparinoid with anti-inflammatory properties and no anticoagulation activity. The drug also have antianaemic effects, as shown by an experimental study in a mouse model of CKD [93]. Recently, phase-1 data from healthy volunters showed that sevuparin at three different doses reduces hepcidin of plasma hepcidin decreases of 30-50% from baseline values (NCT03853421).

Transferrin receptor 2 (TRF2) could be also a possible therapeutic target; its major function is sensing iron in the hepatocites and control hepcidin production [94]. It also promote cell survival and differentiation of erythroid precursors. Antisense oligonucleotide were developed and tested in a mouse model of knoutout TRF2 [95]; their future application seems challenging since both hepatic and erythroid receptor should be inactived to obtain the anitanemic effect [94].

A possible further approach is interfering with interleukins. Ziltivekimab, an anti-interleukin 6 (IL-6) ligand antibody, has been tested in a phase 1/2 study for anaemia treatment in haemodialysis patients with promising results [96]. According to an exploratory analysis of the Effect of Ziltivekimab on Determinants of Hemoglobin in Patients with CKD Stage 3-5: An Analysis of a Randomized Trial (RESCUEa), a phase-2 trial of 264 non-dialysis CKD patients with increased CRP values, ziltivekimab signficantly increased Hb levels from baseline at week 12 compared to placebo [97]. This went together with increases in serum iron, total iron-binding capacity, and transferrin saturation. A larger study is ongoing primarily aimed at testing the efficacy of drug in reducing hard endpoints in patients with CKD, cardiovascualr disease and inflammation (NCT05021835). A similar study is ongoing in patients with heart failure and inflammation (NCT05636176). Preclinical data also showed possible anti anaemic effects of P2D7KK, an anti-IL-1β monoclonal antibody, in a mouse model of IL-1β receptor antagonist knockout with inflammation and CKD [98]. A

large phase-3 study is ongoing testing Ziltivekimab in patients with CKD, cardiovascular disease and inflammation (NCT05021835).

Table 1. New treatments of possible interest for CKD patients with anaemia

Agent	ClinicalTrials.gov	Company	Mechanism of action	Development phase	Setting	Notes
Agents stimulating erythropoiesis						
Pegmolesatide	NCT03903809	Hansoh Pharmaceutical, China	Small peptide stimulating the EPO receptor	Phase 3	ND and DD CKD and dialysis	-
Luspatercept	NA	Bristol Myers Squibb, USA	Targeting the SMAD2 and SMAD3 signaling	Approved for clinical use	Beta thalassemia or myelodysplastic syndromes	No clinical development for CKD
Targeting the growth of early progenitor cells						
Thrombopoietin	NA	Merck, USA	Increase early bone marrow progenitors to counterbalance their depletion due to intensive ESA use	Preclinical for anemia. Available for clinical use in China. Thrombopoietin receptor agonists used worldwide	EPO-resistant anaemia in rats	Combination therapy with ESAs
Targeting serum hepcidin						
NOX-H94	NCT01372137, NCT02079896	NOXXON Pharma, Germany	Spiegelmer of the molecule	Preclinical, phase 1 and 2	Chronic inflammation and dialysis Patients With ESA-hyporesponsive Anemia	No further development
PRS-080	NCT03325621	Pieris Pharmaceutical, USA	Anticalin antibody	Preclinical, phase 2a	Cynomolgus monkey, haemodialysis	No further development
Ab12B9m	NA	Amgen, USA	Anti hepcidin antibody	Preclinical	Cynomolgus Monkeys	No further development
LY2787106	NCT01340976	Eli Lilly, USA	Anti hepcidin	Phase 1	Patients with cancer	No further development

			antibody		and anaemia	
KY1070	NA	Kymab, UK	Anti- BMP 6 antibody	Preclinical	Rodent models of ACD	No further development
CSJ137	NCT02570854	Novartis, Switzerland	anti/BMP6 antibody	Phase 2	Haemodialysis patients with anaemia	Unpublished data
h5F9.23, h5F9-AM8	NA	NA	Anti-RGMc/HJV	Preclinical	Mouse and a rat model of ACD, Genetic mouse model of IRIDA	No further clinical development
Momelotinib	NA	GSK, UK	JAK1/2 and ALK2 receptor	Approved for clinical use	Myelofibrosis	No data in CKD
Sevuparin	NCT03853421	Novo Nordisk, Denmark; Modus Therapeutics, Sweden	Heparinoid	Preclinical	CKD mouse model of high hepcidin anemia, healthy volounters	Phase 2 planned
LY2928057	NCT01991483	Eli Lilly, USA	Inhibition of hepcidin binding to ferroportin	Phase 2	Haemodialysis	No further clinical development
Targeting Transferrin receptor 2						
Anti-sense oligonucleotide	NA	Ionis Pharmaceutical, USA	To reduce hepatic Tfr2	Preclinical	Mouse model of Anemia of chronic inflammation	Transient improvement of anaemia
Targeting Interleukins						
Ziltivekimab	NCT03926117, NCT02868229	Novo Nordisk, Denmark	Anti-interleukin 6	Phase 1 and 2	Non dialysis CKD and haemodialysis	Phase 3 (ZEUS trial, NCT05021835; HERMES, NCT05636176)
Clazakizumab	NCT03744910	Bristol Myers Squibb, USA; Alder Biopharmaceuticals, USA	Anti-interleukin 6	Phase 3	Chronic active antibody-mediated rejection in kidney transplant recipients; ESKF	Phase 2b/3, study on CV Outcome in DD CKD (NCT05485961)

P2D7KK	NA	NA	Anti-interleukin 1β	Preclinical	RaKO mice with CKD	NA

RGMc/HJV, repulsive guidance molecule c/hemojuvelin; ACD, anaemia of chronic disease; IRIDA, Iron-refractory iron deficiency anaemia; CKD, chronic kidney disease, ESKF, end-stage kidney failure; CV, cardiovascular; Tfr2, Transferrin Receptor 2; JAK, Janus kinase; ALK; DD, dialysis dependent; ND, non-dialysis dependent.

CONCLUSIONS

Iron and ESAs play an essential role in the treatment of anaemia, particularly in the context of CKD, and their importance has always been recognised. Today, we have increasing evidence that iron therapy does not only correct anaemia, but also improves cardiac performance, possibly through better mitochondrial function. Experimental evidence suggests that iron supplementation may also be helpful in correcting macrophage iron status and reducing inflammation and fibrosis in the kidney. However, the presence of inflammation, as in CKD, cardiovascular disease and metabolic syndrome, can alter the functional role of iron and potentially cause tissue damage in certain cell types. Indeed, following increased oxidative stress, intracellular labile iron can become a mediator of cellular depth through ferroptosis. Iron accumulation is also one of the possible pathogenetic mechanisms of atherosclerosis, vascular damage and calcification. Metabolic hyperferritinemia is another example of systemic inflammation causing iron accumulation and predisposing to organ damage.

Taking all this into account, we must be aware that iron is a double-edged therapeutic tool that can cause harm if used too little or too much, and therefore needs to be used judiciously and consciously. Inflammation is probably the key factor in this process.

The advent of HIF-PHI has highlighted the fact that stimulation of endogenous EPO is one of many activities of the HIF system, including control of iron metabolism. Stimulation by PHD inhibition increases iron availability and ameliorates inflammation. However, contrarily to expectations, this new class of drugs has at best shown non-inferiority to ESAs on the risk of MACE.

The development of new agents that target inflammation and its consequences may provide new opportunities not only to improve anaemia, functional iron deficiency and hyporesponsiveness to ESA, but also to reduce the risk of hard endpoints.

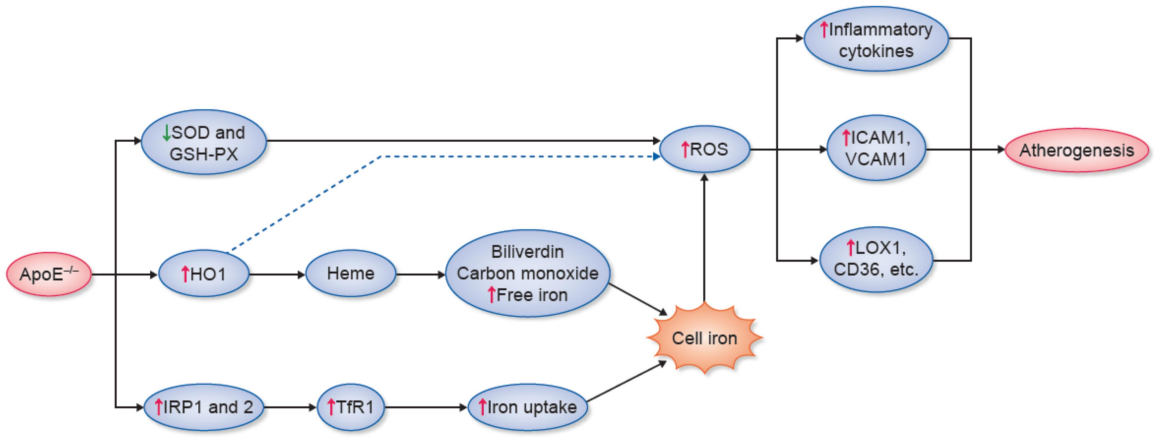

Figure 1. The potential role of iron in the development of atherosclerosis in apolipoprotein E (ApoE) deficiency mice

ApoE might have a negative effect on iron homeostasis by inhibiting the IRP/TfR1 pathway under physiological conditions. In ApoE-deficient mice, this physiologically negative effect disappears, and iron accumulates in the aortic tissues likely via upregulating IRP/TfR1 pathway. Increased ROS production, induced mainly by increased iron and possibly also partly by reduced SOD and GSH-PX enzymes, plays an important role in endothelial activation and consequent monocyte recruitment to the arterial intimae, via upregulation of the expression of cellular adhesion molecules ICAM1 and VCAM1, the main ox-LDL receptor of endothelial cells LOX-1, important receptor for oxidised lipoproteins CD36, NF-κB phosphorylation, and proinflammatory cytokines TNFα, IL-1β, and IL-6, in the aortic tissues of mice. **Abbreviations**: GSH-PX, glutathione peroxidase; ICAM1, intercellular adhesion molecule-1; IRP, iron regulatory protein; LOX-1, lectin-like ox-LDL receptor; 1SOD, superoxide dismutase; TfR1, transferrin receptor 1; VCAM1, vascular cell adhesion molecule-1.

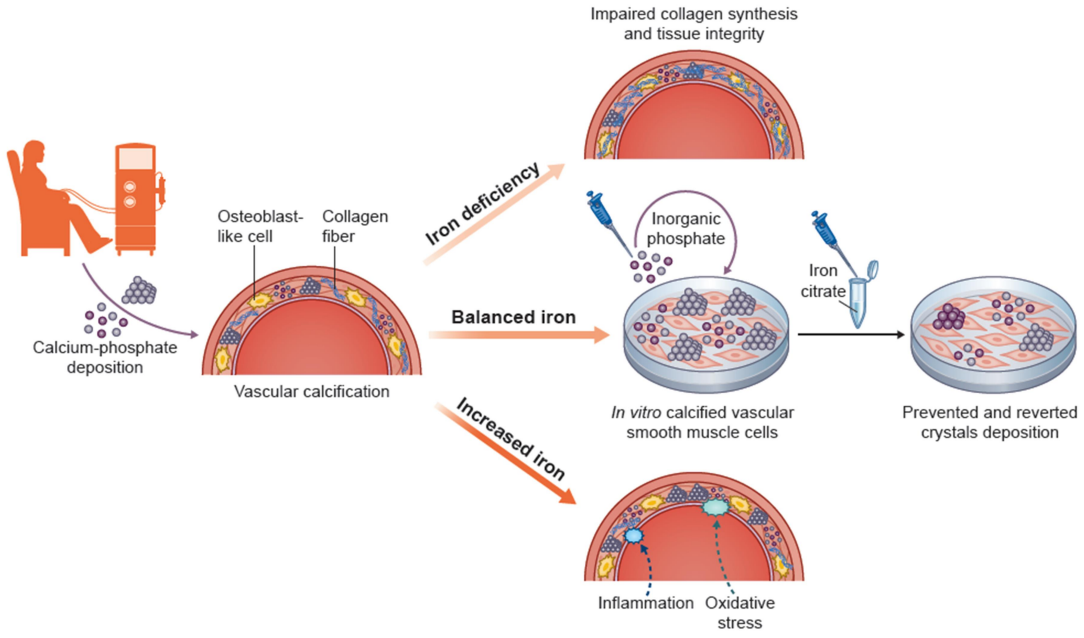

Figure 2. Mechanisms linking iron with vascular calcifications

Representative scheme of the influence of iron on vascular calcification. Iron deficiency can contribute to calcification impairing collagen synthesis and tissue integrity. In an *in vitro* model of vascular smooth muscle cells phosphate-mediated calcification, iron citrate can prevent and partially revert crystals deposition. Increased iron levels can exacerbate vascular calcification promoting oxidative stress and inflammation.

AUTHORS' CONTRIBUTIONS

All the authors contributed equally to the conceiving, writing, and editing of the review.

CONFLICT OF INTEREST STATEMENT

LDV received speaker fees at meeting with indirect support from Amgen, Astra-Zeneca, Vifor, Bayer, Astellas. She had been member of an Advisory Board for Travere

D.G. Advisory Board: Sanofi, Kedrion-Pharmacosmos, Vifor Pharma, Novo Nordisk.

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C.Q, Q. G. Z.M.Q. and P.C. have no conflict of interest to declare.

DATA AVAILABILITY STATEMENT

No new data were generated or analysed in support of this research.

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