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Full title: Iron and Platelets: a subtle, under-recognized **relationship**

Short title: Iron and Platelets

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1. INTRODUCTION

The key role of iron in **the production and functioning of** red blood cells has been **long** established (1), **but** iron **also** plays a **similarly** important role for white blood cells (2). The present review aims to **provide an update of findings on** the interactions between iron and platelets. These interactions, long observed by clinicians, have **recently** benefited from important advances **that improve our understanding of** their **physiology** and **pathophysiology**.

2. IRON AND PLATELETS: **GENERAL ASPECTS**

2.1. The platelet: an anucleated but sophisticated blood cell (3, 4)

2.1.1. Morphological and functional aspects. Platelets (or thrombocytes) are cytoplasmic fragments derived from bone marrow megakaryocytes **and** produced at a daily rate of approximately 10^{11} . Once released into the plasma, they circulate, at a concentration of $150-400 \cdot 10^9/L$ **and have** a physiological lifespan of approximately seven to ten days. They undergo progressive senescence, particularly marked by desialylation of the platelet surface glycoproteins (5), before being cleared by the macrophagic system, especially in the spleen and liver. Platelet life span is regulated by a finely tuned process, ensuring that 10^{11} platelets are removed daily (6). Platelet structure and composition reflect the numerous facets of **their** activities. They contain mitochondria, mRNA and all the translational machinery needed for protein synthesis (**approximately** 5000 proteins are present inside platelets (7)), including **the** ribosomes, endoplasmic reticulum, and Golgi apparatus. They host several types of storage granules containing a wide array of potentially active molecules (fibrinogen, growth factors, cytokines, chemokines, adenosine diphosphate (ADP), serotonin) (8), and secrete a myriad of membrane vesicles (both exosomes and microvesicles) **into the blood which together** represent the

majority of circulating vesicles (9). Moreover, platelets **have adhesion** and immune receptors (10, 11). They are also able to generate reactive oxygen species (ROS) especially as products of the electron transport chain (**ETC**), and it is important to **remember** that ROS exert a *physiologically* pivotal role, **as they fine-tune** several signaling pathways (12-14).

2.1.2. Pathophysiological aspects. Platelets are involved in both beneficial and deleterious processes. **While** the most classical beneficial effect is their crucial function in hemostasis through clot formation, their appropriate positioning at the endothelial sites following vessel injury **also puts** platelets **on the front line for** sensing and counteracting pathogens. Thus, activated platelets are able to induce innate (natural) immunity (**15**) by releasing antimicrobial proteins, chemokines and cytokines, and by recruiting neutrophils and monocytes/macrophages; platelets can also interact with the adaptive (acquired) immune system through crosstalks with B and T lymphocytes. From a detrimental **point of view**, hyperactivation of platelets can cause thrombus formation in atherosclerosis diseases or in inflammatory/infectious diseases (16), and excessive immune-related platelet activation can lead to thrombocytopenia as observed during autoimmune diseases (e.g. systemic lupus erythematosus) (17).

2.2. Role of iron in platelet physiology

2.2.1. Role of iron in platelet formation

According to the classical view of hematopoiesis, thrombopoietin controls megakaryopoiesis and thrombopoiesis (18) and platelets originate from bipotent megakaryocytic/erythroid progenitors (MEPs)(4, 19). Several genetic or acquired factors, independent of thrombopoietin (20), **direct** the lineage commitment of MEPs toward either megakaryocyte progenitors (MkPs) or erythroid progenitors (ErPs). Genetic **determinism of these processes** has been suggested by²genome-

wide association studies that have identified **specific polymorphisms that impact both red blood cell and platelet production** (21, 22). Among acquired factors, iron status has recently been reported as critical for regulating the lineage commitment of MEPs. Using acquired (low iron diet) and genetic (*Tmprss6*^{-/-}) mouse models of iron deficiency developing thrombocytosis, Xavier-Ferruccio et al (23, 24) demonstrated that lack of iron led to a preferential megakaryocytic commitment of MEPs. From a mechanistic viewpoint, the following **characteristics** were observed in **MEPs**: low intracellular labile iron, low ERK (**extracellular signal-regulated kinase**) signaling, and slow proliferation. Moreover, these findings were concordant with *human* primary MEPs in *in vitro* experiments: after **they had** been depleted of their iron sensor TFR2 (transferrin receptor 2), the cells recapitulated the megakaryocytic commitment bias observed in the above-mentioned *Tmprss6*^{-/-} mouse model (23). Although cellular iron content of those human MEPs was not investigated, the authors considered that disrupting the TFR2 iron-sensing mechanism would **mimic** a low-iron environment. This may appear paradoxical **because, in** humans (25) and mice (26), TFR2 loss of function causes marked systemic iron overload. **Nevertheless**, it has been shown that *specific* bone marrow TFR2 loss of function stimulates erythropoiesis without causing systemic body iron excess (27). Furthermore, using a systemic TFR2 deficient mouse model, Wortham et al recently showed that extrahepatic (bone marrow and spleen) TFR2 deficiency was associated with increased erythropoiesis, independently of iron overload (28). The preferential orientation, in iron deficiency, toward the production of platelets instead of red blood cells remains to be explained. **This** regulatory mechanism may, on a chronic basis, preserve the use of iron for functions other than erythroid differentiation and, in acute clinical situations, help stop a life-threatening hemorrhage (24).

2.2.2. Role of iron in mature platelets

Because platelets contain no nuclear genomic DNA, the major role of iron in DNA synthesis (29) does not apply to them **at the nuclear level, although it could play a role in mitochondrial DNA synthesis**. The lack of nucleus may have opened the window for improved platelet functional efficiency (30), and, given the role of iron in many cellular processes, significant functional interactions between iron and platelets **would be** expected (Fig.1).

- Iron and platelet **mitochondria**

The number of mitochondria in each thrombocyte is relatively low (less than ten). However, their functional efficiency **at coping** with intense activity requiring **high** energy (ATP) production **is remarkable**. This energy-consuming activity is notably related to granule secretion and platelet flexibility, **i.e., the capacity to change from a resting state to an activated one** and vice versa. It has been shown that **ATP production in platelet mitochondria is more efficient than ATP production in mitochondria of monocytes and lymphocytes** (14, 31, 32). Knowing that ATP is derived from glycolysis and oxidative phosphorylation and that iron is involved in both processes (33), **throws light on** interactions between iron and platelet mitochondria. Whether platelet mitochondria play a significant role in heme synthesis, iron-sulfur cluster biogenesis, **or** ferritin synthesis (34, 35) remains to be explored; **this is also the case** for mitochondrial iron trafficking (36) and **any** possible interactions between iron, platelet mitochondrial ferritin and mitophagy (37).

- Iron trafficking and metabolism in platelets (**other than** mitochondria)

It is quite plausible that most platelet iron comes from the megakaryocytes themselves and that their original iron load is sufficient to meet the needs of the platelets during their short life. **There are no reports, to our knowledge, documenting the presence of ferritin within the platelet cytosol and data**

remains fragmentary on the role of potentially "active" iron actors at the platelet level itself. **Thus, the presence of TFR1** (transferrin receptor 1), **which** is the cellular "entrance gate" for plasma transferrin-iron, **is debated. Its expression was not found in human platelets by Hannuksela et al (38),** unlike the cellular iron sensor TFR2 or the HFE protein (38, 39), **but has been reported by the platelet proteome-data base (40).** It, therefore, remains to be shown **whether** classical TFR1-iron endocytosis (41) **is functional in platelets.** If this is the case, it could open the possibility of a mechanism for iron delivery from the endosomes to the mitochondria either via a cytosolic pathway or, as proposed by Hamdi et al in reticulocytes, through direct interaction between endosomes and mitochondria ("the kiss-and-run" hypothesis") (42). It should be asked **whether TFR2 could also be involved in entry into platelets and its delivery to mitochondria (41).** The presence of non-transferrin bound iron transporters (43) **has not yet been confirmed. Ferroportin,** the cellular iron exporter (44), **has been detected in the platelet proteome-database (40) but its functional activity has not yet been documented.** Although the expression of hepcidin, the iron hormone (45-47), has not been reported in platelets, it should be mentioned that the platelet-derived growth factor-BB (PDGF-BB), a potent angiogenic and chemoattractant agent (48) **produced by platelets (but also by other cell types),** has been shown to mediate hypoxia-induced downregulation of hepcidin by hepatocytes , increasing the availability of plasma iron for erythropoiesis (49).

3. IRON AND PLATELETS: CLINICAL AND MECHANISTIC ASPECTS

3.1. IRON DEFICIENCY AND PLATELETS

3.1.1. IMPACT OF IRON DEFICIENCY ON PLATELETS

Table 1 presents the different effects of iron deficiency on platelets.

3.1.1.1. ABSOLUTE IRON DEFICIENCY

Absolute iron deficiency (ID) may be defined as a decrease in total body iron stores and can lead to iron deficiency anemia (IDA). The main causes of **absolute** iron deficiency are iron losses of gynecological (menorrhagia), digestive (peptic ulcer, colon cancer), or blood donation origin (50); or decreased duodenal iron intake (51, 52), due to an **iron-poor diet**, coeliac disease, long term use of proton pump inhibitors, or iron refractory iron deficiency anemia (IRIDA)(53).

- Thrombocytosis (platelet count above $400 \cdot 10^9/L$) is a classical clinical feature in chronic iron deficiency, described as one of the **conditions** of "reactive" thrombocytosis (54). If we consider only **absolute** iron deficiency (i.e., excluding iron deficiency related to inflammation, see below), reactive thrombocytosis is **has been observed at the following frequencies**: 13% in a study of 615 subjects by Kuku et al (55), 28% in 86 women by Kadikoylu et al (56), and almost 33% in a **large population of 36,327 IDA patients** by Song et al (57). As mentioned above, the main mechanism whereby **absolute** iron deficiency favors thrombocytosis could be a shift of MEPs toward MkPs at the expense of ErPs (23). It has also been reported that thrombocytosis in IDA could involve a downregulation of tubulin which is one of the main components of **the** platelet cytoskeleton (58), but **this** may be a consequence **rather than** a causative factor. **Estimates of** the thrombotic risk in the event of thrombocytosis related to **absolute** iron deficiency **differ greatly**: no cases of thrombosis were recorded **by** Kuku et al (55), whereas thrombotic complications were observed by Song et al in 16% of IDA patients with thrombocytosis, compared with 8% in IDA without thrombocytosis (57). Recently, Jimenez et al (59) provided original experimental mechanistic data on the relationship between decreased body iron stores and the development of thrombosis. Using rat models of dietary ID with ⁶venous or

arterial thrombosis, they showed that ID was responsible for thrombocytosis which, in turn, was an independent risk factor for the development of thrombosis. Hypercoagulability was found, together with increased platelet and plasma P-selectin (reflecting hyperactivity), and with impaired platelet adhesion and aggregation under shear flow; importantly, all these changes were reversed by iron supplementation. **It should be noticed that the relationship between iron deficiency and venous thromboembolism (VTE), which was confirmed by a broad-based-population study (60), might involve other factors than thrombocytosis.** Thus, still in the field of iron metabolism, transferrin upregulation could play a significant role as shown by Tang et al (61). These authors found, both *in vitro* and *in vivo*, that ID, estrogen administration, exogenous transferrin or transferrin overexpression induced hypercoagulability and aggravated ischemic stroke. In contrast, anti-transferrin antibodies, transferrin knock-down and designed peptide inhibitors exerted anti-ischemic stroke effects. However, contrary to the view that ID may explain the association between red cell distribution width and risk of VTE, Ellingsen et al (62) reported that increased plasma levels of hepcidin, considered as biomarker of iron stores, were associated with an increased risk of VTE. It should be noted, however, **that variations of both hepcidin and ferritin levels remained minimal.**

- Thrombocytopenia can be observed in severe iron deficiency, and clinicians should be aware of this rare event (less than fifty reported cases (55, 63-66)). It can be falsely diagnosed as an **immune thrombocytopenia** that can be associated with iron deficiency secondary to bleeding (67). Two **factors** may favor the diagnosis of iron deficiency related thrombocytopenia: i) the **severity of** iron deficiency with marked decreases in hemoglobin, MCV (erythrocyte mean corpuscular volume), and ferritin values; and ii) a low

percentage of reticulated platelets (or low immature platelet fraction IPF) which orientates toward a central thrombocytopenia (**a state of impaired platelet production, as opposed to the increased destruction by autoimmune attack as occurring in immune thrombocytopenia**) (66, 68). Moreover, IPF increases under iron supplementation in parallel with reticulocytes and platelet counts (66). The mechanism whereby major ID, in contrast to minor ID, causes thrombocytopenia remains to be **specifically identified** but it is likely that, in this severe clinical **situation**, the erythropoiesis pathway becomes vital and is therefore “preferred” to thrombopoiesis.

3.1.1.2. **FUNCTIONAL IRON DEFICIENCY**

- **Functional iron deficiency occurs mainly in** inflammatory situations in which systemic iron distribution is modified but total body iron stores remain unchanged. Indeed, especially under stimulation of the IL6-STAT3 signaling pathway, hepatocyte hepcidin secretion is increased, leading to low duodenal iron absorption and low splenic iron release, both **of which are** factors causing low plasma iron and low plasma transferrin saturation.
- As a result, iron entry into the bone marrow and erythrocyte production decreased, **contributing, in the long-term, to the development of** anemia of chronic disease. Importantly, the decreased plasma iron compartment is associated with an *increased* macrophagic iron compartment. This macrophagic iron retention (bone marrow, spleen, liver) is related to decreased cellular iron release, itself due to the decrease of ferroportin iron export activity which **results from high** hepcidin level (69-72). On the whole, there is iron misdistribution with normal **overall** body iron stores.
- From the platelet **perspective**, the links between inflammation and iron may **have several important** aspects. On the one hand, it is **expected that**

decreased iron availability to hematopoietic precursors (due to low iron-saturation of transferrin and/or to hepcidin-mediated iron-trapping in bone marrow macrophages) contributes to *thrombocytosis*. The development of ID-related *thrombocytopenia* is however less expected due to less potential ID severity during inflammation than in major **absolute** ID. On the other hand, as mentioned above, inflammation itself can interact with platelets independently of iron. This is especially observed in acute inflammation.

- During infection, thrombocytopenia occurs rapidly due to the extensive platelet consumption that follows the release of immunomodulatory agents and the interaction of platelets with neutrophils to form neutrophil extracellular traps (73). This process may have severe clinical consequences, including increased mortality, due to loss of vascular integrity, hemorrhage, and even septic shock (74). It is followed by a quick regeneration of platelets to counteract this inflammation-induced thrombocytopenia. Haas et al have shown that inflammation drives efficient cell cycle activation and maturation of stem-like megakaryocyte committed progenitors, that rapidly replenishes the platelet pool during acute inflammation (75). Thrombopoietin may be involved in this thrombopoiesis activation mechanism through its stimulation by IL-6 (76).

- Clinically, inflammation-related thrombocytosis is observed in infectious diseases (77) but also non-infectious ones such as inflammatory bowel disease (78, 79), where a correlation has been found between plasma hepcidin levels and platelet counts (80), auto-immune disorders (systemic lupus erythematosus (81), juvenile arthritis (82)), various malignancies including solid tumors (83), or chronic myeloproliferative neoplasms (84), in which thrombocytosis can also, of course, be of primary origin. As for the

thrombotic risk, it may result from the conjunction of reactive thrombocytosis and the natural history of the causal disease (85).

3.1.2. IMPACT OF PLATELET ALTERATIONS ON IRON METABOLISM

Whether of acquired or genetic origin, severe thrombocytopenia or thrombopathy (functional platelet alterations) can lead to hemorrhage and in turn to **absolute** iron deficiency. Moreover, depending on the etiology, inflammation may also interfere with iron metabolism by favoring systemic iron misdistribution. It should be noted that platelet counts alone are a poor predictor of hemorrhagic risk, unless platelet counts are $\leq 5000 \cdot 10^9/L$, as shown in patients with hypoproliferative thrombocytopenia due to stem cell transplants or chemotherapy for malignancy (86).

3.2. IRON OVERLOAD AND PLATELETS

Table 1 presents the different effects of iron overload on platelets.

3.2.1. GENETIC IRON OVERLOAD (HEMOCHROMATOSIS) AND PLATELETS

There are no available data documenting the potential impact of hemochromatosis on blood platelet *counts* (87, 88), unless there is a high degree of hepatic fibrosis (89), possibly related to hypersplenism (portal hypertension) and /or hepatocellular failure (deficient synthesis of thrombopoietin). **It should be recalled, however, that in hemochromatosis, such severe hepatic dysfunction is mostly observed when there are cofactors of liver toxicity (e.g., alcohol, non-alcoholic fatty liver disease).** Regarding platelet *quality*, Mikaelssdottir et al (90) studied, over a seven-day storage period, platelet-rich plasma obtained from 10 newly diagnosed hemochromatosis patients undergoing phlebotomy therapy. They concluded that no differences were observed between the patients and a group of healthy donors, for platelet aggregation (after activation with ADP, arachidonic acid, collagen, or

epinephrine), as well as for expression of surface (CD62P and CD42B) and secreted (sCD62P and sCD40L) activation markers. **These results differ from previous data showing that iron levels found in hemochromatosis patients inhibited γ -thrombin-induced platelet aggregation (91).** Moreover, a recent study (92) of 43 iron overloaded patients, including 19 *C282Y/C282Y* hemochromatosis patients and one case of *HJV* (hemojuvelin) hemochromatosis, reported that plasma transferrin saturation was inversely correlated with platelet reactivity. Iron overload has also been shown to alter mitochondrial respiration in a rat model of hemochromatosis (93), and morphological alterations of hepatocyte mitochondria have been observed in severe cases of human hemochromatosis (94). Moreover, focusing on human platelets and iron exposure, non-heme iron was unable to induce platelet activation, unlike platelets that had been first primed with collagen, in which case total platelet aggregation occurred (95). Hemin induced platelet activation and death through ferroptosis (96) involving C-type-lectin-like receptor-2 (97). It is therefore **possible** that human platelet mitochondria also represent a target of iron-related ferroptosis in hemochromatosis.

3.2.2. ACQUIRED IRON OVERLOAD AND PLATELETS

Chronic acquired iron overload is mainly the result of multiple transfusions and/or ineffective erythropoiesis as occur in β -thalassemias (98, 99), myelodysplastic syndrome (MDS) (100) or following therapeutic **ablation** in bone marrow transplantation (2). Due to platelet changes, whether quantitative (101-103) and/or qualitative (99, 104-106), which can develop as part of the underlying hematological diseases (or of their medications), **it is difficult to attribute** platelet modifications to iron overload alone. One way to approach this issue is to evaluate the impact of iron chelation treatment on thrombocytes. Numerous studies have reported hematological improvement, including⁶ platelet

counts, in MDS patients under oral chelation by deferasirox (107-110). However, most of these studies did not find any significant correlation with decreased body iron stores; only Cheong et al reported, in a prospective, multi-center, open-label study of 96 patients that elevated platelet counts (together with increased hemoglobin) were associated with a significant decrease of both serum ferritin and liver iron concentration (107). These results have led to search for mechanisms other than the iron factor alone that could account for hematological improvement. In vitro studies have identified the impact of deferasirox on ROS (111), NfκB (112), or mTOR (113). Performing genome-wide transcriptomics **in peripheral blood mononuclear cells** of 15 patients with low-grade MDS treated with deferasirox, Sanchez et al (114) identified deregulated genes likely to contribute to hematological improvement: downregulation of genes related to the NfκB pathway, downregulation of γ-interferon, inhibition of NFE2L2/NRF2 that play a key role in reducing ROS, overexpression of miR-125b (micro-RNA playing a key regulator of hematopoietic stem cells), and overexpression of GFI1 (gene growth factor independent 1), which is a transcriptional repressor playing a critical role in hematopoiesis.

4. CONCLUSION

Iron is required for the overall process of hematopoiesis, and fluctuations of the bone marrow iron load environment **affect whether** cells **become** platelets or erythrocytes. A low iron context favors platelet synthesis. Iron is also involved in the mitochondrion machinery which is particularly efficient in platelets. **Absolute** iron deficiency is frequently associated with thrombocytosis **which** may increase the risk of vascular thrombosis. **Thrombocytopenia** can also occur in severe iron deficiency. **Functional** iron deficiency (misdistribution) develops during inflammatory conditions and may be associated with early **thrombocytopenia** followed by rapid replenishment of the blood platelet pool, with sometimes reactive thrombocytosis. Whether chronic iron overload in hemochromatosis **can** alter platelet

functioning with clinical consequences remains to be explored. Oral iron chelation improves platelet blood count in acquired (transfusional and/or dyserythropoietic) iron overload, but without strong evidence of the role of iron depletion itself. It is likely that a disturbing **effect** of the iron chelator on several signaling pathways is the main mechanistic explanation. In order to better understand the clinical interactions between iron and platelets, further basic research is **necessary**, focusing on the putative presence and role in platelets of the various actors of cellular iron transport and metabolism, and keeping in mind that platelet iron may **originate** from the iron contained in their mother cells.

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FIGURE LEGEND

Figure 1. Platelet iron trafficking and metabolism. Mitochondrial iron is critical for ATP production through oxidative phosphorylation (**ETC: electron transport chain**); iron plays also a role in the production of ROS that can, depending on their amount, either tune signaling pathways or exert deleterious effects. Many **questions remain** regarding iron trafficking through platelet membranes, their pathways within the cytosol, and their crossing of mitochondria membranes. NTBI: non-transferrin bound iron; TFR1: transferrin receptor1 **(the question mark indicates that the functional activity of TFR1 has not yet been documented)**; TFR2: transferrin receptor2; **ETC:** electron **transport** chain; ROS: reactive oxygen species; PDGF-BB: platelet-derived growth factor-BB; Fe-S: iron-sulfur clusters. **The numerous question marks emphasize the need for further investigations on the relationship between iron and platelets.**

TABLE 1 Platelet changes in iron deficiency (ID) or iron overload disorders (IDA: iron deficiency anemia)

	Absolute ID (decreased total body iron stores)	Functional ID (inflammation-related iron misdistribution)	Iron overload (increased total body iron stores) <i>Genetic</i> <i>(hemochromatosis)</i> <i>Acquired</i> <i>(thalassemia/MDS)</i>		
Thrombocytosis	++	++	Not documented	+	mainly related to causal disease
Thrombocytopenia	+ (severe IDA)	++ (acute inflammation)	Not documented (except if cirrhosis)	+	
Thrombopathy	+	+	Unlikely	+	

FIGURE 1