Body iron delocalization: the serious drawback in iron disorders in both developing and developed countries

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Over 2 billion people in both developing as well as developed countries – over 30% of the world's population – are anaemic. With the classical preconception that oral iron administration or the intake of foods rich in iron increase haemoglobin concentration and reduce the prevalence of anaemia, specific programs have been designed, but iron supplementations have been less effective than expected. Of note, this hazardous simplification on iron status neglects its distribution in the body. The correct balance of iron, defined iron homeostasis, involves a physiological ratio of iron between tissues/secretions and blood, thus avoiding its delocalization as iron accumulation in tissues/secretions and iron deficiency in blood. Changes in iron status can affect the inflammatory response in multiple ways, particularly in the context of infection, an idea that is worth remembering when considering the value of iron supplementation in areas of the world where infections are highly prevalent. The enhanced availability of free iron can increase susceptibility and severity of microbial and parasitic infections.

The discovery of the hepcidin–ferroportin (Fpn) complex, which greatly clarified the enigmatic mechanism that supervises the iron homeostasis, should prompt to a critical review on iron supplementation, ineffective on the expression of the most important proteins of iron metabolism. Therefore, it is imperative to consider new safe and efficient therapeutic interventions to cure iron deficiency (ID) and ID anaemia (IDA) associated or not to the inflammation.

In this respect, lactoferrin (Lf) is emerging as an important regulator of both iron and inflammatory homeostasis. Oral administration of Lf in subjects suffering of ID and IDA is safe and effective in significantly increasing haematological parameters and contemporary decreasing serum IL-6 levels, thus restoring iron localization through the direct or indirect modulation of hepcidin and ferroportin synthesis. Of note, the nuclear localization of Lf suggests that this molecule may be involved in the transcriptional regulation of some genes of host inflammatory response.

We recently also reported that combined administration of oral and intravaginal Lf on ID and IDA pregnant women with preterm delivery threat, significantly increased haematological parameters, reduced IL-6 levels in both serum and cervicovaginal fluid, cervicovaginal prostaglandin PGF2 α , and suppressed uterine contractility. Moreover, Lf combined administration blocked further the shortening of cervical length and the increase of foetal fibronectin, thus prolonging the length of pregnancy until the 37th–38th week of gestation. These new Lf functions effective in curing ID and IDA through the restoring of iron and inflammatory homeostasis and in preventing preterm delivery, could have a great relevance in developing countries, where ID and IDA and inflammation-associated anaemia represent the major risk factors of preterm delivery and maternal and neonatal death.

Keywords: anaemia, iron deficiency, lactoferrin, pregnancy, malaria, iron homeostasis, inflammatory homeostasis

Iron requirements

Iron, an essential element for cell growth and proliferation, is a component of fundamental processes such as DNA replication and energy production. However, iron can also be toxic when present in excess because of its capacity to donate electrons to

oxygen, thus causing the generation of reactive oxygen species (ROS), such as superoxide anions and hydroxyl radicals.¹ ROS are known to cause tissue injury and organ failure by damaging a number of cellular components, including DNA, proteins and membrane lipids. This dichotomy of iron, able to gain and lose electrons, has led to the evolution of tight controls on iron uptake to minimize iron deficiency as well as iron excess. Sophisticated

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strategies have been also developed to bind and store elemental iron in a nontoxic, readily available form.

The total body iron, about 3 g in women and 4 g in men, is mainly incorporated as haemic-iron in the haemoglobin, myoglobin and cytochromes (2–2.7 g), and as non-haemic form in various enzymes. In humans, iron absorption occurs in the proximal small intestine (duodenum) in which dietary iron is daily absorbed, ensuring iron in the bone marrow. A typical diet in developed countries provides about 15 mg of iron per day but only about 10%, corresponding to 1–2 mg, is absorbed due to its exceptionally poor bio-availability. Every day, macrophages recycle 20 mg of iron derived primarily from lyses of senescent erythrocytes for the *de novo* synthesis of haem. The iron released from the catabolism of senescent erythrocytes appears to be the largest source of iron in the reticuloendothelial system. Finally, few milligrams of iron are daily recovered from storage in hepatocytes and macrophages.

Iron homeostasis

It is important to underline that iron status is traditionally classified as either iron deficient or iron replete purely on the basis of the estimated total amount of body iron. This is a hazardous simplification as it completely neglects the distribution of iron in the body. The correct balance of iron, defined iron homeostasis, involves a physiological ratio of iron between tissues/secretions and blood, thus avoiding its delocalization. Iron homeostasis guarantees that quantity and distribution of iron is adequate to the requirements.

Cellular iron homeostasis

Cellular iron homeostasis assures that amount of iron taken up by cells is appropriate to the requirement to avoid the deficiency or overload of this important microelement. The great majority of body cells acquire iron via binding iron-saturated transferrin [(Tf-Fe(III))], present in extracellular fluids and plasma, to the Tf receptor 1 and 2 (TfR1 and TfR2), with subsequent endocytosis.² TfR1 is expressed at high levels on erythroid precursors but also on virtually all dividing cells, while TfR2 is predominantly expressed in liver cells and binds serum Tf (sTf) at lower affinity than TfR1. Both diferric and monoferric Tf traffic into endosomes where the low pH strips the iron from the receptor-ligand complex. The TfR1-Tf is cycled back to the plasma membrane, where at neutral pH, the iron-free Tf is released to plasma and TfR1 becomes available for the next cycle of iron uptake.² In the endosomes, the released ferric ions are reduced by a ferrireductase to ferrous ions, which are then transported across the endosomal membrane into cytoplasm via Divalent

Metal Transporter 1 (DMT1).³ In the cytoplasm, a portion of the iron is stored by ferritin (Ftn), a protein composed of 24 subunits endowed with ferroxidase activity, able to harbour up to 4500 iron atoms per molecule as oxy-hydroxide micelles.⁴ Iron export is assured by ferroportin (Fpn), a protein with a molecular weight of 67 kDa and 12 putative transmembrane domains.⁵

Cellular iron levels modulate the synthesis of several proteins involved in iron metabolism as TfR1, DMT1, Ftn and Fpn through the regulation of translational mechanisms. This regulation occurs through the interaction between iron-regulatory proteins 1 and 2 (IRP1 and IRP2) and iron-regulatory elements (IREs) in messenger RNAs (mRNAs) encoding proteins involved in cellular iron homeostasis.⁶ The binding of IRPs has opposite effects on protein synthesis depending on the location of the target IREs that form characteristic stem-loop structures either in the 5' or in 3' region. The binding of IRPs to 5' IRE mRNAs inhibits translation thus decreasing protein synthesis, while the binding to 3' IRE stabilises mRNA and increases protein synthesis by preventing endonucleases from cleaving sensitive regions of the mRNA.^{7–9} Therefore, cellular iron homeostasis is maintained via the IRP/IRE system by modifying the production of proteins involved in cellular iron uptake, storage and export: iron deficiency increases cellular iron uptake, mobilizes iron from cellular storage and decreases Fpn translation, while, when physiological intracellular iron concentration is restored or it is in excess, the IRP/IRE system increases cellular iron storage to prevent accumulation of toxic forms of iron and its export by Ftn. The Figure 1 summarizes the cellular iron homeostasis mechanisms.

The IRE/IRP system also coordinates the synthesis of haem, iron-sulfur clusters, and ferroproteins with the availability of iron. The erythroid cells also possess a mechanism to counteract iron deficiency. Of note, haemoglobin, α -globin, β -globin, and haem are synthesized in proper stoichiometry to form stable haemoglobin. Haem-regulated translation mediated by the haem-regulated inhibitor kinase (HRI) provides one major mechanism that ensures balanced synthesis of globins and haem. HRI is activated by cellular stress as nutrient deprivation, viral infection, and endoplasmic reticulum stress. HRI phosphorylates the α -subunit of translational initiation factor 2 (eIF2 α) in haem deficiency, thereby inhibiting protein synthesis globally. In this manner, HRI serves as a feedback inhibitor of globin synthesis by sensing the intracellular concentration of haem through its haem-binding domains.¹⁰ Therefore, HRI is essential for the regulation of globin gene translation and the survival of erythroid precursors in iron/haem deficiency.¹¹

Cells acquire iron via binding iron saturated transferrin -Tf-Fe(III)- to the Tf receptor 1 and 2 (TfR1 and TfR2). Tf-Fe(III) traffic into endosomes where the low pH strips the iron from the receptor-ligand complex. The TfRs-Tf is cycled back to the plasma membrane, where at neutral pH, the iron-free Tf is released to plasma and TfRs become available for the next cycle of iron uptake. In the endosomes, the released ferric ions are reduced by a ferrireductase to ferrous ions, which are then transported across the endosomal membrane into cytoplasm via Divalent Metal Transporter 1 (DMT1). In the cytoplasm, a portion of the iron is stored by ferritin (Ftn) and exported by ferroportin (Fpn). Cellular iron levels modulate the synthesis of TfRs, Ftn and Fpn through the regulation of translational mechanisms.

Figure 1 Cellular iron homeostasis and translational regulation.

Systemic iron homeostasis

The systemic iron homeostasis is tightly controlled. Both iron absorption by enterocytes and iron recycling by macrophages are highly regulate through iron absorption, storage and export, as mammals lack a regulated pathway of iron excretion.^{12,13} Absorption of nearly all dietary iron (1-2 mg daily) takes place in the proximal duodenum and includes the reduction of iron from the ferric (III) to the ferrous state (II) by a ferrireductase (duodenal cytochrome B, DCYTB) and the transport into the cells by DMT1.³ Interestingly, it has been demonstrated that DMT1 is required for intestinal iron transport or delivery to erythroid precursors.¹⁴ Iron absorbed by DMT1 enters the cytosol of enterocytes where it can be stored by Ftn or exported into plasma by the basolateral iron exporter Fpn.⁵ As reported, the synthesis of Fpn is modulated by IRE/IRP system. In conditions of low iron levels in the enterocytes¹⁵ or during inflammation and after lipopolysaccharide administration,¹⁶ IRP binds to IRE to inhibit the translation of Fpn, resulting in a decrease in iron export from the enterocyte to the circulation.

Fpn, the only known cellular iron exporter from tissues into blood, has been found in all cell types involved in iron export, including enterocytes, hepatocytes, placental cells.¹⁷ This iron transporter

acts in partnership with the hephaestin, a ferroxidase present in enterocytes that oxidizes exported ferrous iron to facilitate its binding to sTf. Fpn has been also found in macrophages where plays a major role in exporting iron released by the degradation of haem out of the cell through the ceruloplasmin, another ferroxidase which oxidises the iron from the ferrous to the ferric form before it is bound to sTf.^{18,19}

Once in the portal circulation ferric iron chelated by sTf is transported to sites of use (erythroid precursors) and storage (hepatoocytes).

Iron export by Fpn is regulated by hepcidin, another pivotal component of systemic iron metabolism. Hepcidin is a peptide hormone containing 25 amino acids synthesised by hepatocytes as a precursor containing 84 amino acids. The bioactive human hepcidin was first identified in human urine^{20,21} and plasma.²² Hepcidin regulates the entry of iron into plasma also through the binding and degradation of Fpn.^{23,24} Conflicting data are reported on the Fpn degradation mechanism. The mechanism of Fpn internalization has been first understood to require JAK2-mediated phosphorylation of Fpn tyrosine residues 302 and 303²⁵ and a transcriptional response initiated by Janus kinase(JAK2)-mediated phosphorylation of signal transducer and activator of transcription-3 (STAT3).^{26,27} Recently, in vitro results demonstrated that hepcidin-induced Fpn internalization did not

require JAK2 or phosphorylation of Fpn residues 302 and 303, nor did it induce JAK-STAT signalling.²⁸ Moreover, it has been further demonstrated that hepcidin binding causes rapid ubiquitination of Fpn, thus suggesting ubiquitination is the functionally relevant signal for hepcidin induced Fpn endocytosis.²⁹ Fpn degradation hinders iron export thus enhancing the cytosolic iron storage. Thus hepcidin is a dominant negative regulator of plasma iron, which also induces the macrophageal degradation of Fpn resulting in iron trapping into macrophages.³⁰

Hepcidin synthesis is suppressed by anaemia and hypoxia while it is up-regulated in response to iron loading and inflammation.^{31–33} Hepcidin mRNA, suppressed during hypoxia, decreased hepcidin synthesis thus resulting in increased iron export into the plasma.³² Recently, the decrease of hepcidin appears as an indirect effect dependent on increased erythropoietin production and expansion of erythroid precursors in the marrow and not as a direct effect of hypoxia-regulated pathways on the hepcidin promoter.^{34–36} In healthy volunteers, the administration of erythropoietin was sufficient to lower serum hepcidin within less than 1 day, in the absence of any significant changes in serum iron.³⁷ In dialysis patients, erythropoietin therapy decreased serum prohepcidin levels while significantly increased haemoglobin and haematocrit.³⁸ A decreased hepcidin expression has been related to anaemia.³⁹ In clinical trials on anaemic pregnant and non-pregnant women, low levels of serum prohepcidin have been found.⁴⁰ Hepcidin production appears to be suppressed when iron stores are low, and consequently, Fpn can be displayed on basolateral membranes of enterocytes restoring iron transport to plasma.

Conversely, when iron stores are adequate or high, iron load increases hepcidin transcription through the canonical bone morphogenetic protein (BMP) pathways.³⁰ Accordingly, the synthesis of prohepcidin increased together with the increase of haematological parameters in pregnant women.⁴⁰

Noteworthy is the hepcidin synthesis markedly induced by an inflammatory status. In particular, interleukin-6 (IL-6) induces transcription of the hepcidin gene in hepatocytes thus inhibiting iron release from enterocytes and macrophages.³¹ IL-6 activates JAK-STAT3 pathway with STAT3 binding to the hepcidin gene promoter, thereby inducing hepcidin synthesis.^{41,42} The hepatic release of hepcidin can also be induced by IL-1 α and IL-1 β .⁴³ Independent on hepcidin synthesis, high levels of serum IL-6 and intracellular iron deficiency appear to down-regulate Fpn mRNA expression, thus blocking iron flow into plasma and increasing iron sequestration inside cells.^{44,45} The Figure 2 summarizes the systemic iron homeostasis and its disorders.

Increased hepcidin and decreased Fpn gene expression result in decreased plasma iron concentration and increased intracellular iron sequestration.

Iron homeostasis in pregnancy

The pregnancy is an emblematic physiopathological condition characterised by an increased iron requirement, due to enhanced blood volume and development of foetal-placenta unit.⁴⁶ Iron transfer from the mother to the foetus is supported by a substantial increase in maternal iron absorption during pregnancy and is regulated by the placenta.^{47,48} Most iron transfer to the foetus occurs after the 30th week of gestation and likely involves placental expression of those proteins known to mediate systemic iron homeostasis. The placental syncytiotrophoblast acquires ferric iron bound to Tf at the apical membrane through TfR-1.⁹ The synthesis of placental TfR-1 increases in pregnant women suffering of ID or IDA,⁴⁸ providing a suggestion of why the degree of foetal ID is not always as severe as that in mother.⁴⁹ Likewise, the foetus seems to be the winner in the maternal-foetal conflict also in endemic malaria areas.⁵⁰

Ftn is strongly expressed in the stroma and contributes to iron accumulation in foetal tissues.⁵¹ In contrast, maternal serum Ftn (sFtn) usually markedly decreases between 12th and 25th week of gestation, probably as a result of iron utilisation for expansion of the maternal red blood cell mass. Fpn is also expressed on the placental basal foetal-facing membrane, consistent with unidirectional mother-foetus iron transport.⁵¹ Concomitantly with the enhanced placental-foetal iron transport, controlled by foetal hepcidin, an increased expression of placental Fpn is also observed.⁵¹ However, inflammatory processes related to infections could increase serum IL-6 levels thus promoting hepcidin synthesis. High levels of foetal hepcidin could induce internalisation and degradation of placental Fpn thus inhibiting iron export. Iron retained in the erythrocyte precursor by hepcidin-mediated degradation of Fpn, may well compensate for this decreased iron supply and/or at least partly, protect the foetus against anaemia.⁵²

In light of these observations, the treatment of ID and IDA during pregnancy should be entirely reconsidered taking into account not only the enhanced blood volume and development of foetal-placenta unit, but also the pivotal role that factors regulating iron transport from tissues to blood, i.e. Fpn and hepcidin, might play on putative adverse events of pregnancy.

Iron homeostasis disorders

Iron deficiency and iron deficiency anaemia

Excessive iron absorption results into iron-overload in parenchymal tissues, while low iron absorption

Iron homeostasis (left panel): iron absorption takes place in the proximal duodenum and includes the reduction of iron from the ferric (III) to the ferrous state (II) by a ferrireductase (duodenal cytochrome B, DCYTB) and the transport into the cells by Divalent Metal Transporter 1 (DMT1). Iron absorbed by DMT1 enters the cytosol of enterocytes where it can be stored by ferritin (Ftn) or exported into plasma by the basolateral iron exporter ferroportin (Fpn). This iron transporter acts in partnership with the hephaestin, a ferroxidase to facilitate ferric iron binding to serum transferrin (sTf). Fpn has been also shown in macrophages where plays a major role in exporting iron released by the degradation of haem out of the cell through the ceruloplasmin, another ferroxidase which oxidises the iron from the ferrous to the ferric form before it is bound to sTf. Iron homeostasis disorders (right panel): hepcidin, synthesised in hepatocytes, is up-regulated by pro-inflammatory cytokines, iron load, and infectious diseases. Hepcidin inhibits the entry of iron into plasma through the binding and degradation of Fpn.

Figure 2 Systemic iron homeostasis.

leads to plasma iron deficiency, which manifests as ID and IDA. These iron disorders are mainly related to the dysregulation of hepcidin, Fpn, Tf, TfRs, ceruloplasmin or hephaestin synthesis.¹⁴ As already reported, increased hepcidin gene expression and the consequent decrease of Fpn levels result in decreased plasma iron concentration. ID in this instance is characterised by iron retention in the intestinal mucosa and in macrophages. The inability to export iron leads to a decreased pool of sTf-Fe(III) and iron-limited erythropoiesis. In inflammatory disorders and infections, cytokine-induced hepcidin up-modulation as well as hepcidin-independent Fpn down-expression, contribute to the development of anaemia of inflammation, characterised by hypoferremia and anaemia despite adequate iron stores.⁵³

In ID without anaemia, total serum iron (TSI) concentration and sFtn decrease, while haemoglobin (Hb) levels remain normal.

ID may be classified according to sFtn and TSI concentrations (<24 ng/mL and <30 mg/dL, respectively) as mild (sFtn = 12 – 24 ng/mL) or severe ID (sFtn <12 ng/mL). In IDA, the deficit of iron is so severe that iron stores are absent or unavailable resulting in abnormally low Hb and red blood cells (RBCs). IDA may be classified according to the number of RBCs ($<4 \times 10^6$ cells/mL) and to the Hb concentration (<11 g/dL) as mild (Hb 7 – 10.9 g/dL) or severe IDA (Hb <7 g/dL).

ID and IDA are the most common iron disorders in the world which lead serious public health problems.⁵⁴ Over 2 billion people in both developing as well as developed countries – over 30% of the world's population – are anaemic. More generally, when iron requirement is higher than that absorbed, a negative iron balance occurs and iron stores decrease. In low income countries, ID and IDA are mainly due to the low bioavailability of iron in cereal- and tuber-based diets containing high amounts of polyphenols

(tannins) and phytates that inhibit iron absorption. Therefore, ID may be considered as one of the most important signals of malnutrition or 'hidden hunger'.

For infants, the health consequences of ID and IDA include premature birth, low birth weight, high susceptibility to infections, and elevated risk of death. Later, physical and cognitive development are impaired, resulting in lowered school performance.⁵⁵

In pregnancy, the increased iron requirement, due to enhanced blood volume and development of foetal-placenta unit,⁴⁶ generally leads to ID and IDA in both developing and industrialised countries. Recent data show that the prevalence of IDA in pregnant women in industrialized countries is about 17% while the incidence of IDA in developing countries increases significantly up to 56%.⁵⁶ ID and IDA represent an important risk factor for maternal and infant health, and, in developing countries, contributes to 20% of all maternal deaths (World Health Organization).

Iron supplementation

The global scale and magnitude of ID and IDA, combined with health and socioeconomic damages, requires the urgent adoption of effective measures to tackle this critical problem.

With the classical preconception that oral iron administration or the intake of foods rich in iron increase haemoglobin concentration and reduce the prevalence of anaemia, programs on iron supplementation and/or iron food fortification have been designed. Governments and International Agencies have considered both iron supplementation and food fortification programs attractive for their apparent simplicity and cost-effectiveness. However, in practice many such programs are proving to be difficult to manage, more costly than expected to implement, and less effective than promised. Although iron supplementation and/or iron fortified foods are considered logical interventions in subjects suffering of ID and IDA, the discovery of the hepcidin-Fpn complex, which greatly clarified the enigmatic mechanism that supervises the systemic iron homeostasis, should prompt to a critical review of these therapeutic interventions.

It has been reported that iron supplementation generates hydroxyl radical in rats with chronic dietary iron loading,⁵⁷ affects the production of proinflammatory cytokines in IL-10 deficient mice⁵⁸ and aggravates inflammatory status of colitis in a rat model.⁵⁹ Recently, in the clinic, not all anaemic pregnant women responded adequately to oral iron therapy.^{40,56,60} Significant decreases of TSI combined with a dangerous increase of serum IL-6 have been observed in pregnant women treated with oral ferrous sulfate.^{40,61} Oral ferrous sulphate is found to be ineffective against

anaemia in haemodialysis patients⁶² as well as in infants and in women of reproductive age from developing countries.⁶³ On the other hand, it is well known that oral administration of ferrous iron often fails to restore iron homeostasis in patients suffering ID and IDA, and frequently causes several adverse effects as gastrointestinal discomfort, nausea, vomiting, diarrhoea, and constipation.^{60,64-67}

Alternative iron supplementations are represented by intravenous iron administration⁶² or transfusions.⁶⁸ However, also these therapies show significant limits. In particular, the intravenous administration of iron is generally associated with its rapid clearance from the bloodstream after an initial rise, while the transfusions partially relieve the erythropoietin-driven expansion of ineffective erythropoiesis, raise hepcidin concentrations but overall cause iron overload owing to the iron content of transfused blood.

Concerning food fortification programs in developing countries, the efficacy of fortified foods in preventing the deficiency of iron and other microelements in infant and children who did not suffer any parasitic or microbial infections, are inconclusive to cause of several components of these foods as Zn and Fe, which can interfere on mutual intestinal absorption. The effects of fortified milk or cereals have been assessed on 1-4 year-old children of peri-urban community located on the outskirts of New Delhi or on 6-12 month-old infants of disadvantaged urban South African areas.^{69,70} Some Countries, such as Mexico, have introduced country food programs, where fortified milk is one component.⁷¹ However, in all these studies the evidence of the effect of fortified milk and cereals on iron metabolism of infants and children has not been systematically assessed. Recently, a systematic review reports that iron plus micronutrient compared with iron fortified milk and cereal food can be an effective option to reduce anaemia of children up to three years of age in developing countries. However, the evidence for functional health outcomes is still inconclusive.⁷² In other studies, the iron-fortified rice on Brazilian infants (10-23 month-old)⁷³ or the fortification of rolls with microencapsulated iron sulphate with sodium alginate on the haemoglobin levels in Brazilian pre-schooler children (2-6 years) did not show any benefit.⁷⁴

Furthermore, it should be emphasized that iron homeostasis disorders are also frequently exacerbated by infectious diseases particularly in resource-poor areas of low income countries. Malaria, hookworm, schistosomiasis, HIV infections, and bacterial infections as tuberculosis are particularly important diseases contributing to the high prevalence of severe anaemia related to the increased morbidity and mortality in some areas. In this respect, iron

supplementation or iron fortified foods could be considered a reasonable intervention. However, a large iron supplementation trial in Pemba, Tanzania, resulted in increased morbidity and mortality in iron-replete children.⁷⁵ In response to this trial, although the usefulness of iron fortified foods has been previously recognized by World Health Organization (WHO), WHO Expert Consultation advised against universal iron supplementation in malaria-endemic regions without prior screening of individuals for ID (WHO, 2007).

It should be highlighted that the consumption of these foods or iron supplementation can enhance the risk of an iron overload in tissues and secretions thus increasing cell damage, susceptibility and severity of infections especially in developing countries with high incidence of microbial and parasitic infections.⁷⁶

In this respect, clinical studies have demonstrated a strong association between iron status and tuberculosis. In iron-deficient Somali nomads, an increase in infections, including tuberculosis, upon the administration of 13 mg/Kg of oral ferrous sulphate/day for 30 days has been demonstrated.⁷⁷ In another study, increased dietary intake of iron associated to tissue iron overload in some African populations was positively related with morbidity and mortality from pulmonary tuberculosis.⁷⁸ Accordingly, subjects from rural Zimbabwe, acquiring high levels of dietary iron from traditional beer prepared at home, increased iron stores as indirectly detected by sFtn concentration and Tf saturation, thus enhancing the risk of active pulmonary tuberculosis.⁷⁹ The relationship between iron overload and increased host susceptibility to tuberculosis should worry and further lead to a critical analysis on the putative damage of iron supplementation.

Negative aspects of iron supplementation on morbidity and mortality are also emerged in several clinical trials on preschool children carried out of regions where malaria transmission is intense.^{75,80}

However, conflicting data have reported.^{81,82} In particular, Menendez *et al.*⁸² have demonstrated that oral iron supplements were safe and effective in preventing IDA without any significant impact on susceptibility to malaria in Tanzanian infants. This trial has been designed administering, from 8 to 24 weeks of age, oral ferrous iron (2mg/Kg) at lower doses respect to therapeutic doses (13 mg/Kg) found to increase the risk of malaria.⁷⁷

It can firstly be underlined that in addition to the limited characterizations between enrolled subjects (children, infants or adults, anaemic or non-anaemic, infected or non-infected or co-infected, inflamed or non-inflamed) and the geographical regions with different prevalence and transmission of microbial and parasitic infections, the conflicting findings on

positive or adverse effects of iron supplementation may be explained by the critical differences in duration, doses, route of iron administration and concomitant antimalarial or anti-microbial treatment.⁸³ Moreover, the lack of data on haematological parameters (RBCs, TSI, Ftn and IL-6), which define the relationship between ID/IDA and inflammatory processes before and after the treatment, may distort the interpretation of iron therapy effects.

Even if iron supplementation would be highly desirable, facilitating optimal cognitive and physiologic development, and alleviating the risks associated with ID and IDA, its supplementation may have serious adverse consequences on host response to infections especially in areas of high infectious burden. Therefore, currently available data do not support definitive guidelines as to when it is safe and efficacious to administer iron in such settings, particularly in the context of malaria.⁸³

Malaria and anaemia

Plasmodium falciparum still causes upwards of 200 million infections, and nearly 1 million deaths annually, a significant portion of which are children.⁸⁴ This parasite displays a complex multistage lifecycle involving host hepatocytes and erythrocytes. Many factors contribute to malarial anaemia, including haemolysis, increased erythrophagocytosis and suppressed erythropoiesis.⁸⁵ The phagocytosis of infected erythrocytes accompanied by clearance of uninfected erythrocytes, represents an important mechanism of controlling blood trophozoite-stage parasites even if it can provoke a severe anaemia.

Severe malarial anaemia (SMA) is a major life-threatening complication of paediatric malaria characterized by a high TNF/IL-10 ratio. This TNF/IL-10 imbalance could reflect a prevalence of inflammatory phenotype T helper 1 (Th1) respect to anti-inflammatory phenotype Th2, the important source of IL-10 in vivo.^{86–88} The high TNF/IL-10 ratio reflects an insufficient production of IL-10 to prevent or counteract the inhibition of erythropoiesis and the increase of erythrophagocytosis induced by TNF.

It is worthy of interest that blood-stage *P. falciparum* also induces the synthesis of IL-6 in African children.⁸⁹ Children with severe malaria (SM) had significantly higher IL-6 concentrations in plasma than children with uncomplicated malaria (UM).⁹⁰ IL-6 induces an increasing of hepcidin levels in the urine and serum of naturally and experimentally *Plasmodium* infected humans.^{91–93} Urinary hepcidin was not significantly associated with haemoglobin, but was associated with log parasitemia.⁹¹ Even if hepcidin levels are increased during malaria, at least in part due to stimulation of phagocytosis of malaria-infected erythrocytes,⁹⁴ the mechanism of

this elevated hepcidin response has been only recently defined.⁹⁵ In fact, hepcidin concentration in children with SMA was significantly lower than in children with Hb >5 g/dL and significantly falls one week and one month after antimalarial treatment compared with levels on admission. Conversely, TNF and IL-6 and parasite density were significantly associated with hepcidin levels. Their findings support the hypothesis that in malarial anaemia, increased hepcidin levels are related to inflammatory cytokines and malaria parasitemia and consequently, iron is not depleted or absorbed or incorporated into haemoglobin but sequestered, i.e. delocalized, inside enterocytes, hepatocytes and macrophages. If on the one hand hepcidin and IL-6 synthesis and related ID and IDA can be considered as a defence host mechanism to inhibit parasite lifecycle in the blood, on the other hand the increase of iron availability in hepatocytes could contribute to a complete *Plasmodium* sporozoite development. Therefore, iron supplementation in SMA is not only futile and ineffective in increasing haematological parameters but also potentially harmful, because the presence of hepcidin during acute malaria infection further increases iron sequestration into host cells.

Mutations causing alterations in Hb production or structure are known to afford protection against the development of severe forms of malaria. These Hb disorders are present at high frequency in areas where malaria is endemic, indicating a survival advantage for individuals carrying them. However, the protection afforded by haemoglobinopathies against severe forms of malaria has not yet found a definitive answer. Recently, it has been observed that individuals carrying these disorders express low levels of hepcidin. When hepcidin levels are low, Fpn expression in cells is sustained leading to export of intracellular iron, thus avoid iron overload in hepatocytes and macrophages. Importantly, low intracellular iron content may affect activation of innate immune cells leading to diminished production of pro-inflammatory cytokines. Evidence support the notion that development of severe forms of malaria is dependent on immune-mediated damage, caused by unfettered immune responses.⁹⁶

Taken together all the reported data, cytokine- and hepcidin-mediated iron delocalization seems to be an essential mechanism in the anaemia of inflammation, thus playing a pivotal role in the severity of malaria.

Pregnancy, malaria and anaemia

A separate section is worth the problem of malaria and anaemia in pregnant women. In sub-Saharan Africa, approximately 30 million pregnant women are at risk of contracting malaria annually.⁹⁷ During pregnancy, ID and IDA, caused by increased iron

requirements as well as incidence of microbial and parasitic infections in developing countries, represent a high risk for preterm birth, foetal growth retardation, low birth weight, and inferior neonatal health. Moreover, infection- and inflammation-associated anaemia represents a major risk factor of preterm delivery and perinatal mortality.^{98,99}

During successful pregnancies, a shift between inflammatory phenotype Th1 and anti-inflammatory phenotype Th2 with the predominance of Th2 occurs to prevent initiation of inflammatory and cytolytic-responses that might damage the integrity of the placental barrier. Conversely, during infection processes, in response to invading pathogens, Th1 are predominant.¹⁰⁰ It has become evident that Th1 type pro-inflammatory immune responses are essential for controlling the parasite load during the early phase of infection.¹⁰¹ Protective CD4+ T cells release IFN- γ to activate effector cells such as macrophages, which, in turn, may exert anti-malarial effects by releasing TNF.^{102,103} Plasma from healthy and asymptomatic malaria pregnant women were evaluated for biomarkers, associated with Th1 and Th2 cytokine homeostasis. The IL-10 and G-CSF, biomarkers of Th2, were elevated in the asymptomatic group when compared with the healthy group. Thus, asymptomatic malaria carriage may be linked to circulating levels of IL-10 and G-CSF.¹⁰⁴ The balance between the Th1 and Th2 phenotypes influences the pro- and anti-inflammatory cytokine synthesis. A balance between pro- and anti-inflammatory responses may be fundamental to the elimination of the parasite without inducing excessive host pathology as destructive inflammation. Conversely, a lower pro- to anti-inflammatory cytokine ratio, indicates a shift towards a high basal Th2 response, associated to high levels of IL-10 and G-CSF. IL-10 levels measured in maternal peripheral blood mononuclear cells cultured with infected erythrocytes were associated with increased risk of malaria infection in young children. High IL-10 production capacity inherited from parents may diminish immunological protection against *P. falciparum* infection, thereby being a risk for increased malaria morbidity.¹⁰⁵

Moreover, the immunity to malaria appears to be dependent on age. An interesting study has been designed to evaluate the prevalence of *P. falciparum* and anaemia infection in adolescent pregnant girls in the Sekondi-Takoradi metropolis, Ghana. The results revealed that adolescent pregnant girls, still developing their immunity to malaria, were more likely to have malaria infection than the adult pregnant women that have fully acquired immunity to malaria. In addition, adolescent pregnant girls had higher odds of anaemia than their adult pregnant women equivalent.^{106,107}

Understanding of the biological basis for susceptibility to malaria in pregnancy was recently advanced by the discovery that erythrocytes infected with *P. falciparum* accumulate in the placenta through adhesion to molecules such as chondroitin sulphate A. Primigravid women are at increased risk of placental malaria (PM), characterized by the accumulation of *P. falciparum* infected erythrocytes (IE) in the intervillous spaces of the placenta.¹⁰⁸ PM is an important cause of maternal and foetal mortality, and severe sequelae in tropical areas. The pathological alterations of PM consist in the placental sequestration of *P. falciparum* infected erythrocytes and the monocyte accumulation in the maternal intervillous circulation, termed intervillitis,¹⁰⁹ as well as in an increased placental blood TNF concentrations.¹¹⁰ Monocytes and macrophages in the intervillous space frequently contain the malaria pigment haemozoin, and intact IE are also seen within these cells. Proinflammatory cytokines and beta-chemokines are secreted by intervillous macrophages and monocytes in response to IE.¹¹¹ This phagocytosis represents an important mechanism of controlling blood trophozoite-stage parasites, also enhanced by antibody opsonisation.¹¹² However, even if the alteration in cytokine balance is important for clearance of IE from the placenta, it is also dangerously associated with maternal anaemia and preterm delivery.¹¹³ The intervillous space, the main compartment for exchange of nutrients and delivery of oxygen to the foetus, is of utmost importance for foetal development.

The events leading to adverse outcomes of placental malaria can be summarized in four steps: (1) accumulation of *P. falciparum* infected erythrocytes; (2) infiltration of monocytes and macrophages; (3) alteration of the placental cytokine balance and (4) pathogenesis of adverse pregnancy outcomes. These events are triggered by chemokines and cytokines leading to impaired maternal-foetal exchange and damage to the placenta. IL-10 have been detected in PM, and the incidence of mortality has been related to inflammation in the placenta. Even if the additional biomarkers may be required to improve clinical diagnosis and management of malaria during pregnancy, IL-10 may have utility as a biomarker in PM.¹¹⁴ The host mechanisms responsible for suppression of erythropoiesis may involve an excessive or sustained innate immune response or a pathologic skewing of the T-cell differentiation response with the attendant production of certain proinflammatory cytokines.

Other factors, associated with suppression of erythropoiesis and development of SMA, include accumulation of haemozoin, malarial pigment, in bone marrow and altered production of inflammatory

mediators, such as nitric oxide (NO). NO is very important in reducing parasitaemia during the initial phase of blood-stage malaria infection.¹¹⁵ However, iron supplementation inhibits the expression of inducible nitric oxide synthase (iNOS), which subsequently down-regulates the formation of NO in macrophages. NO appears to be critical to macrophage defence against *P. falciparum*.¹¹⁶ As matter of fact, ID may amplify iNOS thus mediating defences against this pathogen. Recently, the decrease of iNOS and NO has been found in macrophages overexpressing Fpn. Overexpression of Fpn significantly impaired intracellular *Mycobacterium tuberculosis* growth during early stages of infection. Enhancing erythropoiesis and production of reticulocytes as well as decreasing iNOS synthesis iron supplementation, and probably increasing Fpn synthesis, might increase host susceptibility to *P. falciparum*.¹¹⁷

Finally, the complex regulatory circuit of inflammation, including IL-6 synthesis, at the maternal-foetal interface, rather than systemic inflammation, appears to play a major role in the aetiology of adverse outcomes of pregnancy.¹¹⁸ Although progress has been made in defining at least some of the factors involved in preterm delivery and birth, there is a pressing need for the identification of the complex regulatory circuits underlying the onset of preterm birth. In particular, the relationships linking preterm birth to selected inflammation-related mechanisms, including Th polarization, iron disorders, and malaria infections remain to be established.

Lactoferrin as modulator of iron and inflammatory homeostasis

Lactoferrin (Lf) is an iron binding cationic glycoprotein of about 690 amino acid residues and MW 80kDa, belonging to the Tf family. Lf is able to reversibly chelate two Fe(III) per molecule with high affinity ($K_d \sim 10^{-20}$ M) and retains ferric iron until pH values as low as 3.0.¹¹⁹ The iron-binding affinity is high enough that, in the presence of Lf, the extracellular iron availability (the concentration of free iron in body fluids) cannot exceed 10^{-18} M, thus preventing the precipitation of this metal as insoluble hydroxides, inhibiting microbial growth and hindering formation of ROS. Lf is highly conserved among human, bovine, mouse, and porcine species.¹²⁰ As is apparent from three dimensional structure of human Lf (hLf) and bovine Lf (bLf), the molecules are folded into two homologous lobes^{121,122} (Figure 3 A and B). The two lobes, connected by a peptide which forms a 3-turn α -helix, are further divided into two domains for each lobe (N1 and N2, C1 and C2). Each lobe binds one Fe(III) ion in a deep cleft between two domains. Iron binding and release are associated with large conformational changes in which Lf adopts

Available from: <http://www.pdb.org/pdb/explore/explore.do?structureId=1LFG>; <http://www.pdb.org/pdb/explore/explore.do?structureId=1BLF>.

Figure 3 Structure of human (a) and bovine (b) lactoferrin in iron-saturated form.

either an open or closed state. The iron-saturated form is closed and much more resistant to proteolytic enzymes than the unsaturated-form.¹²³

Lf is expressed and secreted by glandular epithelial cells and by neutrophils. The highest level (~ 7 mg/mL) is found in human colostrum.¹²⁴ It is also present at lower levels in human mature milk (1.5–4.0 mg/ml) and tears (about 2.0 mg/ml), and at very low levels (< 0.1 mg/ml) in most exocrine secretions as ear wax, saliva, small intestine, vaginal fluid, amniotic fluid, seminal plasma, upper airway fluid, and the cervical mucus. hLf is absent from the lung alveoli¹²⁵ and the skin¹²⁶ while there are no reports of hLf being present in the colon or the urinary tract. This glycoprotein is also present in the secondary granules of mature neutrophils.¹²⁷ Of note, Lf concentration increases in infection and/or inflammation sites due to the recruitment of neutrophils. A total number of 10^6 neutrophils synthesize 15 μ g of Lf.

In the healthy humans, mucosal secretions, which first are injured by microorganisms, have very low iron availability (10^{-18} M) considered as signal of their functional status hindering microbial growth. Conversely, an increased concentration of available iron, as a consequence of some pathologies, favours microbial growth and persistence as well as biofilm formation.^{128–130}

Similarly to several defence proteins and peptides found in the mucosal secretions,¹³¹ Lf is multifunctional glycoprotein with antibacterial, antifungal, antiviral and antiparasitic activity, anti-inflammatory, and immunomodulatory properties.¹³² The first function of Lf, recognized in vitro, was the bacteriostatic activity depending on its ability to sequester

iron necessary for microbial survival and growth.¹³³ Successively, Lf was found to have multiple antimicrobial activities independently on its iron binding properties: i) bactericidal activity through an interaction with surface bacterial structures as lipopolysaccharide and the subsequent permeabilization of microbial membranes and ii) antiadhesive activity by hindering the attachment of microorganisms to the epithelial cells.¹³² The antiparasitic activity dependent on iron chelation has been attributed to this protein.^{134–136} Moreover, Lf interaction with both lipoprotein receptor-related protein and cell surface heparan sulfate, has been in vitro found responsible of the inhibition of *Plasmodium* endocytosis.¹³⁷ In this respect, Lf represents the most relevant protein symbolizing a brick in the wall of natural non-immune defences of human mucosal fluids against microbial infections.¹³²

Of note, a bovine milk derivative Lf (bLf), generally recognized as a safe substance (GRAS) by Food and Drug Administration (USA) and available in large quantities, is utilized in the majority of the in vitro studies as well as in clinical trials to identify putative applications. Similarly to hLf,¹³⁸ even bLf possesses a potent anti-inflammatory activity able to both modulate the inflammatory response by epithelial cells infected by intracellular bacteria^{139,140} and revert/attenuate inflammatory response triggered by Toll-like receptor engagement in antigen presenting cells.^{141,142} The capacity of bLf to reach the nucleus of intestinal cells (Figure 4) and freshly isolated monocytes¹⁴² is comparable to that showed by hLf in endothelial cells.¹⁴³ The Figure 4 shows that, after 1 h of incubation, bLf appears in the apical part of intestinal cell cytoplasm (Figure 4 Panels B-C),

Laser scanner confocal microscopy images of the optical sections recovered to the nucleus level of intestinal cells treated with 0.1 mg/ml of bovine lactoferrin. Panels a and b: bright field and fluorescence microscopy images taken after 1 hour of incubation. Green fluorescent bovine lactoferrin is localized in the apical part of the cytoplasm. In Panel c the enlarged image of the insert in Panel B is shown. Panels d and e: bright field and fluorescence microscopy images taken after 3 hour of incubation. Green fluorescent bovine lactoferrin is localized in the nucleus of the cells.

Figure 4 Localization of bovine lactoferrin in intestinal epithelial cells.

indicating internalization into an endosomal compartment. Within 3 hours, bLf is localized into the nucleus of cells (Figure 4 Panel E). The nuclear localization suggests that this molecule may be involved in the transcriptional regulation of some genes of host inflammatory responses.

Recently, bLf anti-inflammatory activity has been proved pivotal in reverting iron homeostasis disorders in pregnant women suffering of ID and IDA. In more than thousand pregnant women suffering of ID and IDA, oral administration of bLf is safe and effective in both decreasing serum IL-6 levels and increasing haematological parameters.^{40,61,144} The pregnant women received 100 mg of bLf two times a day before meal. A total of 200 mg/day of bLf, iron saturated at 20–30%, supply 70–84 µg/day of iron, respectively. Although the concentration of iron, supplemented by bLf is very far to that daily required, a significant increase of the number of RBCs, the concentration of Hb, TSI, sFt and the % of haematocrit has been detected after 30 days of the treatment. Consequently, the efficacy of bLf in curing ID/IDA is not due to iron supplementation, but to a more complex mechanism of this protein modulating the factors involved in iron homeostasis, including IL-6 synthesis. However, bLf influence on

hepcidin and Fpn synthesis cannot be ruled out. Of note, the first value found to significantly increased already after 15 days of bLf oral administration was TSI. These data provide strong evidence that bLf primarily re-establishes the localization of iron, i.e. the correct iron balance between tissues/secretions and blood increasing Fpn-exported iron with a mechanism dependent or independent on IL-6 reduction. Moreover, after bLf oral administration, the increase of haematological parameters is related to the enhance of prohepcidin levels.⁴⁰ Pregnant women, affected by ID and IDA, before treatment showed high levels of serum IL-6 and low values of serum prohepcidin. The results confirmed bLf's potent effect in both significantly restoring iron homeostasis and haematocrit values and in decreasing serum IL-6, in agreement with data reported in a smaller anaemic pregnant women population.⁶¹ Moreover, bLf significantly increased serum prohepcidin synthesis, simultaneously to the increasing of haematological parameters and decreasing of serum IL-6. Although serum hepcidin detection is considered a much more reliable tool than serum prohepcidin in evaluating iron status, the values of prohepcidin in our clinical trials⁴⁰ are in agreement with those reported by testing serum hepcidin³⁹

Differently from that observed in anaemia of inflammation,^{31,53,145} in pregnant women treated with bLf the increase of serum prohepcidin is not related with an increase in serum IL-6 levels but, conversely, with its significant decrease. Except for inflammation, the molecular pathways underlying regulation of hepcidin are not well understood.

Our data on ID- and IDA-affected pregnant women suggest that prohepcidin serum concentration can vary independent of the inflammatory state, and especially independent of serum IL-6 levels. Moreover, the capacity of bLf to rescue systemic iron homeostasis through Fpn cannot be excluded, in agreement with the data on Fpn down-regulation by IL-6.^{44,45} From this point of view, bLf nuclear localization should be more deeply investigate in vitro to ascertain an its putative transcriptional regulation of hepcidin and Fpn genes involved in the intriguing on-off switch for export of iron.

In the same clinical trials bLf efficacy has been compared with that exerted by ferrous sulphate supplementation.^{40,60,61,144} The pregnant women received a tablet of 520 mg of ferrous sulphate once a day during meals, according to Italian standard practice. The ferrous ions orally administered corresponded to 156 mg/day. The ferrous iron exerted significant less efficacy compared with bLf therapy in curing ID and IDA, and significant increases of serum IL-6 have been observed.^{40,61,144} These results strongly support the possibility that iron supplemented via ferrous sulphate is not exported from cells to circulation, but it is accumulated inside host cells resulting in inflammatory conditions, as demonstrated by the enhance of serum IL-6 levels. During our studies, several pregnant women withdrew from the trial for side effects of ferrous sulphate.^{40,60,61,144} The side effects and the little or no effectiveness of ferrous sulphate have been also found in ID and IDA non-pregnant women. The advances on the down regulation of Fpn by IL-6,^{44,45} may provide an explanation for the failure of ferrous sulphate in treating anaemia in general and specifically in restoring physiological concentrations of haematological parameters.

The adverse consequences of iron supplementation are highlighted not only by our clinical trials in developed Country as Italy,^{40,60} but also in developing Countries, where enhanced availability of free iron increases the risk and severity of microbial and parasitic infections as well as the adverse events, including death.^{75,146,147} On the other hand, if the iron disorders are mainly related to the dysregulation of hepcidin, Fpn, Tf, TfRs, ceruplasmin or hephaestin synthesis,¹⁴ any therapy, including iron supplementation, that is not able to regulate the proteins of cellular and systemic iron homeostasis must be widely revised.

As already reported, ID and IDA in pregnancies significantly increase the risk of preterm birth (PTB).^{98,99} PTB is now viewed as the consequence of pathological signals that activate the physiological pathway of parturition before term. Inflammation at the maternal-foetal interface, rather than systemic inflammation, appears to play a major role in the aetiology of adverse outcomes of pregnancy.¹¹⁸ Although progress has been made in defining at least some of the factors involved in PTB, there is a pressing need for the identification of the complex regulatory circuits underlying the onset of PTB. In particular, the relationships linking PTB to selected inflammation-related mechanisms and iron availability and trafficking, remain to be established. In this respect, a central role for bLf could be envisaged. In fact, bLf is capable to overcome pregnancy-associated anaemia and to modulate the inflammatory response, both phenomena playing an important role in the adverse outcomes of pregnancy.^{61,141}

Recently, we designed an open-label cohort and subcohort study. The cohort was designed to confirm the effect of bLf oral administration on iron and inflammatory homeostasis in anaemic pregnant women. The subcohort including women of the cohort with pre-term delivery (PTD) threat was additionally treated with bLf intravaginal administration. A significant improvement of haematological parameters was observed in the women's cohort together with a consistent decrease of serum IL-6 levels. Combined administration of oral and intravaginal bLf to the women's subcohort with PTD threat decreased IL-6 in both serum and cervicovaginal fluids, cervicovaginal prostaglandin F2 α , and suppressed uterine contractility. bLf intravaginal administration blocked further shortening of cervical length and the increase of foetal fibronectin thus prolonging the length of pregnancy. The deliveries occurred between the 37th and 38th week of gestation.¹⁴⁸ These results provide strong evidence on promising capacity of Lf to reduce the risk of PTB, thus extending the therapeutic potential of this multifunctional natural protein. At long term, this new Lf function could have a great relevance in developing countries, where IDA and infection-associated anaemia represent the major risk factors of PTB.

Conclusions and perspectives

Iron, an essential element for all organisms, is able to gain and loss electrons. This dichotomy has led to the evolution of tight controls on iron uptake, binding, storage and export to minimize the effects of ID as well as iron toxicity when present in excess. To define the iron status neglecting the distribution of this 'nutrilite' in the body is an hazardous simplification. The correct balance of iron, defined iron homeostasis,

involves a physiological ratio of iron between tissues/secretions and blood, thus avoiding its delocalization as hypoferremia in blood and iron accumulation in tissues/secretions. Excessive iron absorption results into iron-overload in parenchymal tissues, while low iron absorption leads to plasma iron deficiency, which manifests as ID and IDA.

In the past decade, the discovery of Fpn-hepcidin complex has greatly helped to define the sophisticated iron homeostasis mechanisms. However, the depth understanding of the regulation of iron metabolism proteins has not been matched to an equally thorough exploration of therapeutic strategies to enhance the effective of iron disorder treatments. As matter of fact, the classical preconception that considers oral or intravenous iron administration a logical intervention to increase haemoglobin concentration and reduce the incidence of anaemia, is still prevalent despite the limited if not the harmful effects of iron supplementations. Furthermore, although the development of anaemia is associated with detrimental effects especially in relation to cardiac function, quality of life, growth and mental development, the significant decrease of iron in the circulation may also harbour some potentially positive effects, especially during infections.

Nevertheless, the reduction of circulating iron can be associated with iron load or overload in macrophages. As a consequence of this iron-loaded, macrophages have an impaired potential to kill various bacteria, fungi, parasites, and also viruses, *in vitro* and *in vivo*. Part of this can be attributed to the presence of iron-overload which inhibits the transcription of iNOS and thus the generation of NO.^{116,149} NO is an essential effector molecule of macrophages to fight infectious pathogens. Iron overload has also negative effects on neutrophil function as iron therapy of chronic haemodialysis patients impaired the potential of neutrophils to kill bacteria and reduced their capacity to phagocyte foreign particles.¹⁵⁰

Changes in iron status can thus affect the immune response in multiple ways, particularly in the context of infection, an idea that is worth remembering when considering the value of iron supplementation in areas of the world where infections such as malaria and tuberculosis are highly prevalent. It should be also emphasized that malaria and bacterial infections as tuberculosis are particularly important diseases contributing to the high prevalence of severe anaemia related to the increased morbidity and mortality particularly in resource-poor areas of low income countries. Therefore, iron supplementation in these diseases is not only futile and ineffective in increasing haematological parameters but also potentially harmful, because the hepcidin-induced inflammation during acute phase of infections further increases iron

sequestration into host cells.¹⁵¹ The clear evidence that supplemental iron increases host susceptibility to infections⁷⁶ as well as inflammation also in developed Countries,^{40,61,144} should prompt to a critical review of this therapeutic intervention. On the other hand, if the iron disorders are mainly related to hepcidin, Fpn, and other proteins involved in iron metabolism as Tf, TfRs, ceruloplasmin or hephaestin up-or down-regulated by anaemia, hypoxia, iron loading and inflammation,¹⁵² any therapy, including iron supplementation, that is not able to regulate the main proteins of cellular and systemic iron homeostasis must be widely revised. For example, iron supplementation can be dangerous in disorders such as Fpn disease, which predisposes individuals to macrophage iron retention, thus increasing the susceptibility to infections by intracellular pathogens.¹⁵³

Considering the central role of the hepcidin- Fpn axis in iron regulation and in the pathogenesis of common iron disorders, it is not surprising that this system has been targeted for drug development. The delineation of the molecular determinants of the hepcidin-Fpn interaction is a worthwhile long-term goal that could also facilitate the development of hepcidin or Fpn agonists and antagonists. Other new tools that target hepcidin regulation include suppression of BMP signalling. Compounds, as dorsomorphin reduce BMP signalling and hepcidin production.¹⁵⁴

Moreover, it is well known that the inflammatory disorders are also associated with increased serum IL-6, which induces up-regulation of hepcidin expression³¹ and the down-regulation of Fpn.^{44,45} Treatment with antibodies against the IL-6 receptor has been shown to reduce hepcidin levels in several patients, suggesting an approach to handling the anaemia associated with the inflammatory disorders.¹⁵⁵

Even if advances in the next few years will lead to a more complete understanding of systemic iron homeostasis, a central role for the iron-binding glycoprotein Lf can be envisaged. In fact, Lf has recently emerged as a pivotal component of iron and inflammatory homeostasis capable to overcome ID and IDA in pregnant and non-pregnant women and to modulate the inflammatory response by significant reduction of serum IL-6.^{40,61,144} Moreover, the direct or indirect (by IL-6) influence of Lf on the expression of hepcidin and Fpn cannot be ruled out. Of note the nuclear localization of Lf suggests that this molecule may be involved in the transcriptional regulation of some genes involved in host inflammatory responses.^{140,142,143}

In this respect, ID and IDA in pregnancy greatly increase the threat of PTB and the inflammation at the maternal-foetal interface, rather than systemic inflammation, appears to play a major role in the aetiology of adverse outcomes of pregnancy.¹¹⁸

Although progress has been made in defining at least some of the factors involved in PTB, there is a pressing need for the identification of the complex regulatory circuits underlying the onset of PTB.

Of note, we recently reported that combined administration of oral and intravaginal Lf to pregnant women with PTD threat, reduced IL-6 levels in both serum and cervicovaginal fluid, cervicovaginal prostaglandin PGF2 α , and suppressed uterine contractility. Moreover, Lf administration blocked further shortening of cervical length and the increase of foetal fibronectin, thus prolonging the length of pregnancy and the deliveries occurred between the 37th and 38th week of gestation.¹⁴⁸ Such novel strategy based on oral and intravaginal bLf therapy could be future therapeutic option which, without maternal and foetal side effects, restores iron export from cells to blood and its physiological localization as well as decreases serum and intravaginal IL-6 levels, thus avoiding the dangerous iron accumulation inside host cells.

These new Lf functions effective in curing ID and IDA through the restoring of iron and inflammatory homeostasis and in preventing PTD, could have a great relevance in developing countries, where ID and IDA and inflammation-associated anaemia represent the major risk factors of PTD and maternal and neonatal death.

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