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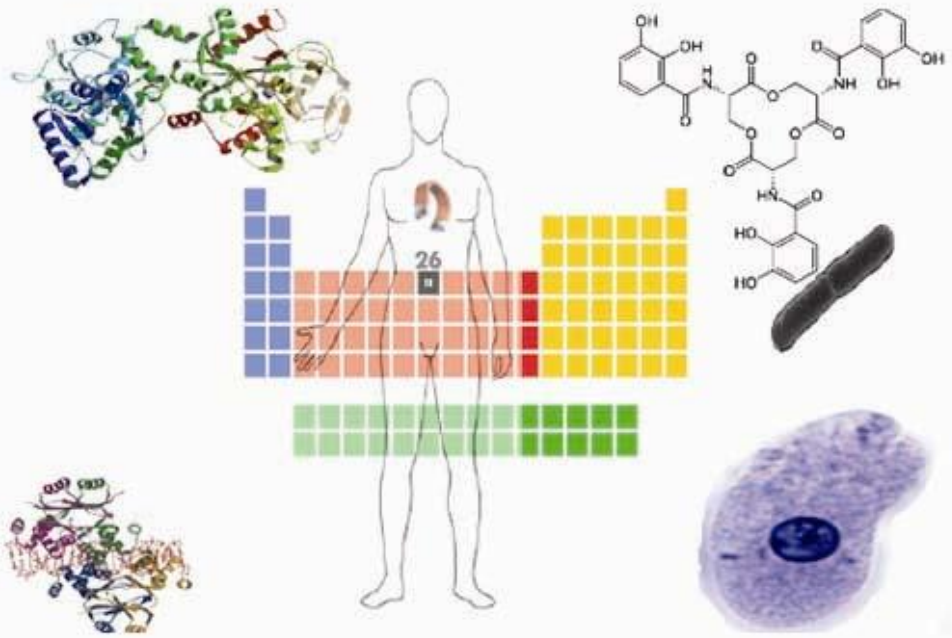
The struggle for iron

pathogen vs. host



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Sergio Vaca Pacheco
(Editors)

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The struggle for iron: pathogen vs. host
First Edition 2013

Editors
Mireya de la Garza Amaya
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PREFACE

Who lives by the sword, dies by the sword

This book is an effort of Mexican researchers who work in the relationship between host and pathogens and the importance of iron in this interaction. It is a new actualized version in English of our previous book "La lucha por el hierro: patógeno vs. hospedero", first published in Spanish in 2010.

Iron is a transition metal essential for near all living beings; it takes part in a great number of vital biological processes. Iron has two oxidation states relevant to its biological functions: Fe^{+2} and Fe^{+3}; the reversible conversion from one to another enables its participation in reactions involving electron transfer. This metal is absolutely necessary for survival and multiplication of protozoa, fungi and bacteria, except the spirochete *Borrelia burgdorferi* and some lactobacilli, which use manganese or copper instead of iron.

Ferrous iron is a reducing agent deleterious for living beings due to it leads to the production of free radicals. Thus, iron is essential but is toxic when in excess, therefore its concentration is strictly regulated. The iron is always bound to proteins (hemoglobin, ferritin, transferrin, etc.). Limitation of free iron avoids multiplication of pathogenic microorganisms inside the hosts. However, microbes have their own mechanisms for obtaining iron, which enable them to overcome this deficiency and to establish an infection.

The aim of this book is to review the iron relevance in host-pathogen interaction. Accordingly, in the first part the physical and chemical properties of iron, its metabolism in the human being, the diseases due to iron-oxidative stress, and the importance of iron storing to avoid its toxicity, are analyzed. In the second part, characteristics of four iron-containing host proteins are described. In the third part, several mechanisms used by pathogens for host-iron uptake, and animal models employed for studying the role of iron on infection, are reviewed.

The purpose of this book is to increase our knowledge about iron and the host-parasite struggle in which pathogens have access to this element. We hope that our book readers become enthusiasts for this fascinating matter.

The editors, May 2013.

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D. OUTLOOK

Chapter 1

Structural organization of iron and its biological function

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Abstract

1. What is what gives "life" to matter?
2. To which group does Fe belong? and In which functions it is involved?
3. What are the special properties that make Fe so effective that no other element can carry out its functions?
4. Why does Fe form stable ions with charges +2 and +3, involving little energy in the transformation of one into the other?
5. Which determines the reactivity of atoms and the number of electrons that change their relative positions, once the reaction has taken place? What commonly happens with Fe?
6. Which of these Fe oxidation states predominates: Fe^{+2} or Fe^{+3}? Are there any other oxidation states?
7. Fe ions: their coordination numbers, "d" electrons, high- and low- spin states, and stability routes.
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9. What determines the lesser atomic radius of Fe? and How does it affect its behavior?
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11. How is the ionic radius of Fe, its solubility and affinity for related ligands?
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Abstract

In this chapter, the biological functions in which iron is involved are presented, along with its crucial role in certain redox processes. It is also highlighted the reason why, by natural selection, the unique organization of Fe makes it an irreplaceable element to almost every living being.

Important concepts that facilitate the understanding of particles organization in iron atoms, the adjustments after iron ionization as well as the interaction of such ions with other ligands are included. Furthermore, it is analyzed the energetic state of the different electron arrangements in order to appreciate the contents of free energy, reactivity and solubility in diverse geometric structures associated with life, in nature. Importantly, it will be assessed the biological functions that take part in the formation of free radicals.

Finally, they are considered the strategies to incorporate and maintain in the biological environment a sparingly soluble ion such as Fe(III), using the much greater solubility of ferrous ion, and preventing the attainment of those levels of this ion, which threaten cell integrity because of its involvement in the generation of free radicals.

Key words: coordination complexes of Fe, degenerate orbitals, ferric hexahydrate, inorganic biochemistry of Fe, redox potential, t2g.

1. What is what gives "life" to matter?

The fundamental difference between living and non-living things lies in their information content. **Information in the universe is modeled by four basic forces, nowadays recognized: gravity force, strong nuclear force, weak nuclear force and electromagnetic force** [1]. The latter one determines the required information for life [2], and shapes electron organization in atoms and their derivatives. According to the structures attained, it can play specific roles.

The electron arrangements with similar organization, behave alike, thus they can be ordered. Life then is produced in the context of the interaction of some members of such groups. According to the viability of the constituent elements of living organisms, they are classified as follows [3]:

1. **Main group (O, C, H, N, P, S)**: In addition to build biomolecules, which is the most important function of this group, these elements help triggering conformational changes. The first four elements listed constitute 99% of the human-body weight (62.8, 25.4, 9.4 and1.4% respectively).
2. **Alkali metals:** They carry out charge-neutralization functions, voltage-gated ion channel activation and structure stabilization.
3. **Alkaline earth metals:** They play the roles of messengers, enzymes activators and participate in structural regulation.
4. **Transition metals:** They are essential in electronic transference, in redox catalysis, as well as in oxygen transportation and storage.

2. To which group does Fe belong? and In which functions it is involved?

Fe (Fe) is a transition metal (these metals have at least an ion with one "**d**" orbital partially filled with electrons) [4]. **Among transition metals, Fe is the most used in electron transfer reactions and in the catalysis of redox reactions** [3].

In the first of these processes the protein function, which prosthetic group contains Fe, is to act as a vehicle for the electron transference, and recovering its initial state after participating in a complete cycle (without a net change). In the second process, the transferred electrons by protein Fe-containing prosthetic group complex promotes a change in the agents that participate in the reaction, as it occurs in the cytochrome oxidase, which transforms O_2 in H_2O.

Along with the referred functions, **Fe has the role of oxygen transport** (as a component of hemoglobin or its analogue pigments), **oxygen storage** (as a part of myoglobin), it also participates in the **biosynthesis of DNA** (catalyzing the formation of deoxy-ribonucleotides via ribonucleotide reductase; in xenobiotic metabolism [5,6] it forms part of hydrolases [3]. In addition to the proteins that have already been

mentioned, Fe is a **constituent of** other proteins equally important, such as **oxygenases**, **flavoproteins** and **redoxins** [6].

Fe distribution in the different biological components illustrates most of its functions. In humans, it has been estimated that average adults have nearly 4 g of Fe, **65%** of them are associated with oxygen transport as a constituent of hemoglobin; **9%** is involved in oxygen storage forming part of **myoglobin;** less than 1% is found in heme containing enzymes. Finally, between 15 and 30% of Fe is stored in soluble form as **ferritin** or in deposits as **hemosiderin** [7]. It is considered that hemosiderin is a protein that results from lysosomal degradation of ferritin and, with the exception of Fe overload, it represents a much lower concentration than that of ferritin.

The irreplaceable role of Fe permits these functions to be performed; therefore, it is remarkable to consider that **virtually all organisms from Archea** (a primitive group of prokaryotic organisms distinct from conventional bacteria) **to human beings depend on Fe for survival** [8].

3. What are the special properties that make Fe so effective that no other element can carry out its functions?

The understanding of the biological function of Fe means to be aware of certain characteristics of this element; such as those described below:

1. **Fe forms ions with oxidation states +2 and +3**, which are stable, though easily convertible due to the relatively low energy required to transfer an electron from Fe^{+2} or towards Fe^{+3}, from oxidant or reducing substrates common to living things.
2. Fe and Manganese (Mn) are the smaller atoms among transition elements, considering their covalent radius. This condition let Fe to form small ions with **d** orbitals that makes Fe a **strong acid (Fe^{+3}) or an intermediate acid (Fe^{+2})**, allowing it to accept electron pairs of diverse ligands which, without changing its ionic nature, affect its trend to receive or donate electrons.
3. **Fe is the most abundant element of our planet,** and it is the 4th most abundant in the Earth's crust (50 000 ppm), after oxygen (460 000 ppm), silicon (277 200 ppm) and aluminum (81 300 ppm) [9].
4. **Fe is able to respond to its surrounding, differentiating the energetic content of its d orbitals originally degenerated** (they are named in this way because in the absence of external influences, there is no energy difference between them). In this way Fe is able to form, together with geometric arrangements and interaction options with different ligands complexes, highlighting structures such as proteins. They provide microenvironments that confer them **redox potential values** (that measure the facility of giving or accepting electrons), covering a highly important range to its amplitude and limit values. The redox potential of the main reducing biologic molecules (like NADPH with -320 mV) and that of the oxidants (like dioxygen molecule with 820 mV) limit the extremes of this range. Table 1 includes the most significant processes in this context.

4. Why does Fe form stable ions with charges of +2 and +3, involving little energy in the transformation of one into the other?

The understanding of the unique qualities of Fe involves the concepts that explain the relationships between the organization of particles in space (mainly electrons that constitute matter), and how they affect each other; above all, the protons on electrons and the electrons on other electrons. These effects define its position according to its energetic content and to the routes towards free energy decrease.

The comprehension of the electrons organization by their energetic content is fundamental to understand the reason why, by natural selection, Fe's properties make it an irreplaceable element to almost every living being, owing to life-essential electron transfer and redox catalysis.

It is important to remember that were it not for the evolution of the systems that balance the Fe intakes, the very nature of this element might have prevented the establishment of life; on one hand **extreme insolubility of Fe^{+3} could compromise its availability**, but on the other hand, **Fe overdose favors free radicals production.**

The participation of Fe^{+3} [also called Fe(III)] in electron transference, leads to Fe(II) formation and therefore "labile Fe" accumulation in cytosol and then Fe can donate electrons to different ligands, for instance H_2O_2, transforming them in **very toxic free radicals** [6].

The most distinctive properties of Fe compared to other elements, are the proton number in its nucleus, and the number and distribution of its electrons. In this respect, **it is very important to recognize that electrons, when moving, occupy spaces determined by the probability of finding them, in turn determined by the energy contained in them.**

The main quantum number (n) indicates how many nodes a particular electron has; **there are only two kinds of nodes: radial and angular**.

The arrangement of the radio nodes is defined by a radius limit to the nucleus of the atom; beyond which the probability of finding electrons becomes zero. On the other hand, angular nodes are characterized by constituting a crosswise plane the center of the atom with no probabilities of finding electrons there. Electrons, when moving across the space contour the series of spots where it is possible to find them, according to the type and number of nodes that each of them presents.

Table 1. REDOX POTENTIALS OF IONIZED Fe IN DIFFERENT FORMS (pH 7, 25 °C), INCLUDING SOME COMPLEXES OF BIOLOGICAL RELEVANCE AND SOME SUBSTRATES THAT OXIDIZE OR REDUCE THEM.

It can be appreciated its important variability [3].

REACTIONS IN WHICH DIFFERENT SUBSTRATES CONNECTED WITH THE BIOLOGICAL FUNCTIONS OF FE ARE REDUCED	POTENTIAL (V) OF REDUCTION WITH RESPECT TO NORMAL ELECTRODE OF H
$\frac{1}{2} O_2 + 2H^+ + 2e^- \longrightarrow H_2O$	+ 0.816
$Fe^{+3} + e^- \longrightarrow Fe^{+2}$	+ 0.771
Cytochrome a3-Fe^{+3} + 1 e^- ⟶ Cytochrome a3-Fe^{+2}	+ 0.550
Fe-prot of high potential(HPAP)-$[Fe_4S_4]^{+3}$ + 1 e^- ⟶ HPAP-$[Fe_4S_4]^{+2}$	+ 0.370
Cytochrome a-Fe^{+3} + 1 e^- ⟶ Cytochrome a-Fe^{+2}	+ 0.290
Cytochrome c-Fe^{+3} + 1 e^- ⟶ Cytochrome c-Fe^{+2}	+ 0.260
Metahemoglobin-Fe^{+3} + 1 e^- ⟶ Hemoglobin-Fe^{+2}	+ 0.139
Cytochrome b- Fe^{+3} + 1 e^- ⟶ Cytochrome b- Fe^{+2}	+ 0.060
Metmyoglobin-Fe^{+3} + 1 e^- ⟶ Myoglobin-Fe^{+2}	+ 0.046
Rubredoxin- Fe^{+3} + 1 e^- ⟶ Rubredoxin- Fe^{+2}	- 0.060
Cytochrome c-Fe^{+3} Peroxidase + 1 e^- ⟶ Cytochrome c-Fe^{+2} Peroxidase	- 0.194
Cytochrome P-450-Fe^{+3} + 1 e^- ⟶ Cytochrome P-450-Fe^{+2}	- 0.300
$NAD^+ + H^+ + 2e^- \longrightarrow NADH$	- 0.320
$NADP^+ + H^+ + 2e^- \longrightarrow NADPH$	- 0.324
Ferredoxin-$[Fe_4S_4]^{+2}$ + 1 e^- ⟶ Ferredoxin-$[Fe_4S_4]^{+1}$	- 0.400
Ferredoxin II-$[Fe_2S_2]^{+2}$ + 1 e^- ⟶ Ferredoxin II-$[Fe_2S_2]^{+1}$	- 0.432

The outermost electrons of the Fe atom are 4s, which have four nodes in total (moving in level n = 4). These electrons do not have sufficient energy to move that distance with angular nodes, all four nodes are radial, which is the combination of lower energy (most stable) of all the electrons that can move with four nodes in total.

The probability of finding these electrons draws a spherical shape called subshell. This form has a single orientation (contains only one orbital). **Fe has electrons that do have angular nodes, including some that are found on level 3. All three level electrons move with 3 nodes in total.** Some have three radial nodes (called **3s**, which also have a spherical shape) and some others have two radial nodes and one angular node (called **3p**, with a two-lobular form) and finally the **3d** electrons have a radial node and two angular ones. The five **3d** orbitals are shown in Figure 1, with a different color per orbital. It can be seen that **most of them are tetra-lobular**, except for the purple one.

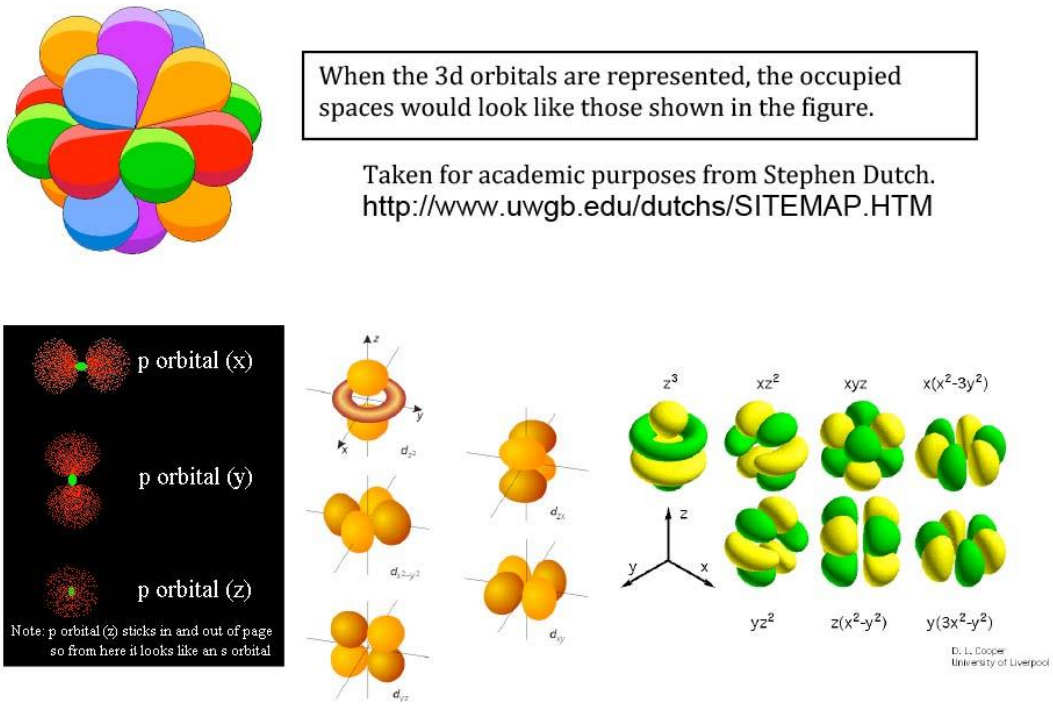


Figure 1. DIFFERENT ORBITAL SHAPES WITH ANGULAR NODES, WHICH HAVE MORE THAN ONE ORIENTATION IN SPACE.

Among all electrons belonging to level 3 of Fe (a total of 14 electrons), there are six electrons with more energy than the rest in that level. These have more angular nodes than the rest (one radial and two angular nodes) and they are called "**3d**". The energy subshell of electrons is characterized by the number of angular nodes (in this case, there are two angular nodes, so the quantum number "l" is 2.

As angular nodes increase, the shape turns more asymmetric, and consequently, there are more possible orientations; each orientation constitutes an orbital. **The shape depicted in space by electrons that do not have angular nodes is spherical, and it has only one orientation; all electrons having one angular node in its movement will describe a bilobular shape, giving rise to three possible orientations.**

All those electrons with two angular nodes can move in any of five possible orientations (these variants are illustrated in Figure 1 and Table 2). Each orbital can admit a maximum of 2 electrons, one of them must attract the other with its magnetic field, to counteract their electric repulsion, because of their negative charge and the short distance between them (as they are in the same orbital), so they must have opposite spins.

The previous explanation emphasizes that there are conditions that set the placement of electrons in an atom. **In essence, electrons move in those available spaces with lower energy**. One Fe atom accommodates 26 electrons around its nucleus; first those spaces with lower energy content will be filled, and once they are occupied, the rest of the electrons will fill the other empty spaces, following an increasing energy content order. (In Table 2, occupied orbitals with the 26 electrons of Fe atoms are indicated in the cells with yellow background). **The first two electrons of Fe occupy level 1,** this level has electrons with only one radial node (the outer edge of the area colored in orange); since there are no angular nodes, there is only one orientation, that is one orbital, this space is called **1s** (shown in the first row of the table).

The next eight electrons occupy level 2 (second row of Table 2), this level contains electrons with two nodes. If both are radial nodes, the energy content of the electrons is the lowest of that level; they are named **2s**, and are only two, for that reason the **2s** subshell has only one orbital. If one of both nodes is radial and the other one is angular, the electrons have more energy, so they move in the **2p** subshell. This subshell has six electrons with three orientations (orbitals).

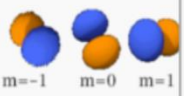
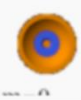
The shape of **2s** orbitals is illustrated in the first column, second row of Table 2, the outer edge of the orange sphere is a first radial node, and the outer edge of the purple colored zone is the second radial node. The remaining six electrons have an angular node and a radial node (see second row, second column of Table 2, the angular node is recognized between orange and purple lobes, the radial node is located in the outer edge limit of both lobes).

At levels 1 and 2, there is only space for 10 electrons, therefore electrons 11 and 12 must occupy level 3. They have opposite spins, three radial nodes and they move in **3s** subshell, with only one orientation (first column, third row in Table 2). **At level 3, there is a second way for having three nodes** (two radial and one angular node). This condition can be reached with three different orientations; each one may contain no more than two electrons (Fe electrons 13 to 18).

Finally, in this same level, there is a third way of having three nodes (one radial and two angular nodes) known as **3d** subshell (column 3, row 3 in Table 2). Subshell **3d** has higher energy than subshell **4s**, it means that after **3p** is filled with electrons, then the next subshell in which the electrons will be is **4s** subshell, no matter if there are still five empty orbitals in level 3. As it can be seen, a movement with two angular and one radial node requires more energy than that with 4 radial nodes. **Thus, in Fe, the 4s orbital fills with electrons 19 and 20 and the electrons 21 to 26 move in orbitals of the 3d subshell.**

Table 2. SHAPE OF ORBITALS IN EACH SUBSHELL AND DISPOSITION IN SPACE OF THOSE LEVELS THAT ARE FILLED MORE FREQUENTLY WITH ELECTRONS

The highlighted-yellow boxes show the orbitals containing Fe electrons.

	s (l=0)	*p* (l=1)	*d* (l=2)	*f* (l=3)
n=1				
n=2				
n=3				
n=4				
n=5				. . .
n=6			. . .	. . .
n=7		. . .	. . .	. . .

The closest electrons to any atom that approaches to Fe are the **4s** electrons, which are not the most energetic, but they are the outermost electrons. These electrons, as already mentioned are 2. The 6 electrons in subshell **3d** are distributed in five orbitals, which cover the entire inner space neighboring electrons **4s** (the space occupied by these orbitals is illustrated in Figure 1).

The lack of gaps and the negative charges saturation cause a repulsion effect among them and make them very available to other positive charges that move toward these electrons, especially to ligands that combine two properties: to have a good number of positive charges in their nucleus and to have a reduced set of electron shells, enough not to interfere approaching ligands. One of these ligands is oxygen, which easily combines with Fe.

The O_2 attacks the **4s** electrons and can even take 1 of the 6 electrons moving in **3d** subshell, thus achieving a more homogeneous distribution of the magnetic force in the space surrounding the Fe, which helps gaining stability.

The stability achieved is because before the sixth electron in Fe is missing, it is experiencing an asymmetry in the distribution of the magnetic fields caused by the existence of a magnetic field-free zone. Full occupancy of one of the five **3d** orbitals, determines the mutual neutralization of the magnetic force associated with the spin of both electrons in that orbital. The magnetic force is evident in the remaining 4 orbitals in **3d** subshell, because these four electrons move with parallel spin. This stability in the magnetic field is reached when a subshell is half filled.

5. Which determines the reactivity of atoms and the change in their relative position once the reaction has taken place? What commonly happens with Fe?

Once the concepts that determine the arrangement of electrons around atoms are understood, especially in Fe, then it should be recognized which factors determine that those electrons reach more stability. In this sense, it is very illustrative to observe a group of elements that under normal conditions do not react; such group is called **noble or inert gases**. The extreme stability of these gases is attributed to their outer energy levels and subshells full of electrons (achieving a homogeneous distribution of negative charge in all the surrounding spaces and neutralizing their magnetic fields in every orbit available).

Elements different from inert gases spontaneously react to acquire an electronic structure, similar to that of a noble gas. **The electrons move spontaneously to those spaces in which their charge and the neighboring charges find their most stable position;** being so that, the most available of those electric forces would propel the others to move more energy and also to reduce the tendency to change when their magnetic fields are neutralized, that is, when available orbitals are filled. **Those elements which manage to resemble noble gases, by giving up or receiving a few electrons, tend to be reactive.** Especially if after the reaction the final positions of electrons, help them to move using less energy than before.

Fe could get the electronic structure of argon if it gives 2 electrons up from subshell **4s** and 6 electrons from subshell **3d**; **the transfer of 8 electrons is an uphill process that cannot occur spontaneously,** given that 8^+ charges are such an intense charge in a very small space that drives to

instability. **Accordingly, only two electrons are usually transferred (instead of eight) from Fe to the other atoms;** level 4 gets empty and leaving the **3d** subshell with 6 electrons.

Each electron moves with the same spin direction, attending to **Hund's rule**, hence the charge distributes homogeneously in the whole space increasing the stability with respect to Fe^{+2}; since the resulting magnetic fields are not neutralized, as they are in krypton atoms; **the stability of Fe^{+3} is unattainable due to the effect of five little magnets that lead to a paramagnetic behavior;** unlike krypton, that having no electrons free is diamagnetic and thus its electrons do not divert their course, under the influence of an external magnetic field. The electronic configurations mentioned above will be as follows:

Configuration of Fe

$1s^2 2s^2 2p^6 3s^2 3p^6 3d^6 4s^2$

Configuration of Ar

$1s^2 2s^2 2p^6 3s^2 3p^6$

Configuration of Kr

$1s^2 2s^2 2p^6 3s^2 3p^6 3d^{10} 4s^2 4p^6$

Configuration of Fe^{+2}

$1s^2 2s^2 2p^6 3s^2 3p^6 3d^6$

Configuration of Fe^{+3}

$1s^2 2s^2 2p^6 3s^2 3p^6 3d^5$

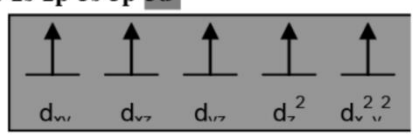


Figure 2. CONFIGURATIONS OF Fe AND ITS IONS COMPARED TO Ar and Kr.

The gray box shows with high detail the subshell d of Fe^{+3}.

6. Which Fe form will predominate: Fe^{+2} or Fe^{+3}? Are there other states of oxidation?

The specific electron arrangements that Fe can take, are affected by the repulsion or attraction of electrons when transferring from one atom to another. Such conditions determine the two most common oxidation states of Fe; Fe(II) or ferrous ion, and Fe(III) or ferric ion. These two forms could be described as two, and three positive charges, respectively. **The intensity of these positive charges varies depending on the ligands that approach to them** (the ligands have a differential capacity to neutralize given by nature and the distance separating them) and for that reason, the effective charge varies.

The Fe that initially lost its two 4s electrons forming the ferrous ion (Fe^{+2}), can lose a third making ferric ion (Fe^{+3}); leaving the subshell 3d half full with 5 electrons. Having five not-full orbitals (see description below) Fe can interact in coordination with a higher number of ligands. It is clear that if there are no Fe atoms or derivatives to interact with, the formation of ferrous or ferric ions is not possible.

However, there are cases where other atoms or chemical groups can attract Fe electrons with a strong force; so during electron transferring, such particles may occupy positions with a lower energy level

(in comparison with those in Fe). Although the greatest stability is achieved in Fe^{+3}, if the oxidizing agent (which takes away electrons from Fe) is a limiting reactant, or if around there are groups with a higher tendency to oxidation (by reducing others), the reduction potential will be transferred to Fe^{+3}; then Fe will predominate in the Fe^{+2} state.

It should be noticed that there is not a significant difference between these two states of Fe oxidation (Fe^{+2} or Fe^{+3}), and on the other hand, an oxidizing or reducing environment thrust the predominance on either of these ions, thus their transition easily occurs in either of the two ways.

The biological environment is able to provide the conditions (place and moment), to achieve the reducing power that makes Fe^{+2} the predominant ion; as well as to reach a sufficiently oxidizing environment to make Fe^{+3} the most abundant ion. In other words, **the biological environment is able to regulate the abundance of each of these ions.**

The stability of a particular oxidation state results from a balance between the ionization energy required to remove electrons from valence orbitals (in the case of Fe: the two **4s** electrons and those in the **3d** subshell) **and the change in molar enthalpy of solvation or ligand association enthalpy** (the heat released or absorbed at constant pressure when a mole of ions, produced by Fe, are surrounded by water molecules or the corresponding ligands).

Fe is a transition element located on the IV row of the Periodic Table. These elements have moderate or low oxidation state, while those which occupy the V and VI row reach higher oxidation states (above +3). **Fe ions can form covalent bonds** "**σ**" **as long as these ions have space in their orbitals and ligands that provide electrons.**

Not every transition element attains this condition; however, **Fe atoms have available orbitals that allow establishing bond ligands up to coordination number 6** (which is the most common case), while 4 and 5 may occur though less frequently. **In its ion form, Fe has six orbitals in the valence shell, able to carry out σ bonds** (these orbitals are **4s**, **4px**, **4py**, **4pz**, **$3dz^2$** and **$3dx^2$-y^2**). The environment required to promote the higher oxidation states (over +3) in the transition elements demand highly oxidizing ligands, not commonly found in biological environments; by similar reasons, oxidation states below or equal to 1, are not typically found in the transition metals that make up living structures [10].

7. Fe ions: their coordination numbers, "d" electrons, high- and low- spin states, and stability routes.

The Fe electronic structure formerly described, lead us to note that it gains stability by losing two electrons; still it is possible to lose a third electron, and as it gets to fit in less-energetic spaces its stability increases. The

slight energetic difference between both stable arrangements, mentioned before, is the main responsible condition that allows Fe to act as a catalyst in reactions involving electron transference.

The great variety of biological processes in which Fe participates (either as ferrous or ferric state) is understood, not only because of its capability to shift from one ionic form into the other, but also by the effectiveness to reach diverse redox potentials to interplay with a range of oxidizing or reducing agents.

Fe stands out from other transition elements because its capability to differentiate the energy content in its **3d** orbitals (only four out of all elements included in the ten columns of the **d**-block, share such quality) besides of having up to **5d** unsaturated orbitals. This differentiation is a consequence of the nature and proximity of the ligands that are coordinated to Fe ions, the coordination geometry of the complex [11], and the oxidation state reached by Fe.

The metal ions that act as **strong Lewis acids like Fe^{+3}** are smaller, more compact and less polarizable ions than others; they prefer strong ligands or strong Lewis bases. On the other hand, **Fe^{+2} ions are halfway between those that constitute strong and weak acids; these ions prefer less compact and more polarizable ligands.**

In addition to the characteristics aforementioned**, ligands preferred by Fe^{+3} present the following conditions: high electronegativity, difficult oxidation and high energy orbitals**; for instance: **H_2O, OH^-, CO_2^-, CO_3^{2-}, NO_3^-, PO_4^{3-}, $ROPO_3^{2-}$, $(RO)_2PO_2^{2-}$, ROH, RO^-, R_2O, NH_3, RNH_2, Cl^-.**

Ligands that prefer Fe^{2+}, considering their intermediate force as Lewis acids are: NO_2^-, SO_3^{2-}, Br^-, N_3^- and imidazole. If the Fe^{+3}, that is constituting some complex, is reduced to form Fe^{+2}; ligands containing oxygen (strong bases) that originally were coordinated to ferric ions are destabilized and can be substituted with ligands containing sulfur or nitrogen, due to the higher affinity of ferrous ions for weak ligands [12]. The reverse process is also possible (with Fe^{+2} oxidation).

The capacity of Fe atoms to change the energy content of their bonding orbitals, considering the aforesaid variants, is evidenced in the series of values of redox potentials that extend in a range of more than 1.0 Volts [13], as are shown in Table 1. This variability is largely related to the spin configuration of their **3d** electrons, as a result of diverse environments generated by the different ligands associated [14].

The six **3d** electrons in Fe^{+2} require the same energy to occupy any of the five orbitals in this subshell, in the absence of external influences. Two of these orbitals are located on the axes of Cartesian plane: **$d_x2_{-y}2$** and **d_z2** orbitals. The three remaining orbitals are located along the Cartesian axes, so their names: **d_{x-y}**, **d_{x-z}** and **d_{y-z}** (the orientation of these orbitals is illustrated in Figure 1).

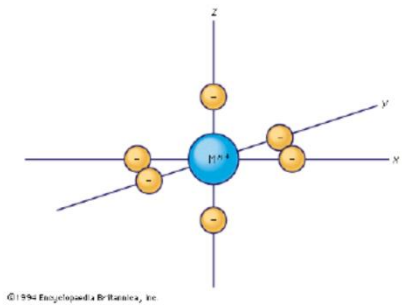


Figure 3. DISPOSITION OF NEGATIVE CHARGES ON AND ABOUT CARTESIAN COORDINATION SYSTEM AXES

If strong Fe ligands approach towards the Cartesian axes (as is illustrated in Figure 3) in the same direction they are arranged, cause a very high energy split among **3d** orbitals (as explained below). The energy of the electrons in $\mathbf{t_{2g}}$ (these three orbitals are located between Cartesian axes, are triple degenerated and present center asymmetry) becomes less significant compared to the energy of those upon the Cartesian plane axes (see orbitals orientation in Figure 3), such electrons are located in $\mathbf{e_g}$ orbitals, (these two orbitals are double degenerated, it means, the four electrons that move inside them have the same energy).

The filling of the five orbitals in this situation leads to a low spin configuration. If ferrous ion is considered, the 6 electrons moving in **3d** subshell fill the three orbitals with low energy, those located between the Cartesian plane axes, neutralizing their spins and the other two **3d** orbitals remain empty. **These empty orbitals can form σ bonds when receiving electron pairs from ligands associated with Fe in coordination 6.**

If ligands are weak, the energetic difference induced by them to **3d** orbitals is lower, favoring electrons to move with parallel spins, leading to a high spin configuration; thus the 6 electrons of the subshell **d** are located as follows; 3 at each of the three low-energy orbitals, 2 at each of the two high-energy orbitals, and the 6th electron goes to any of the first three orbitals [15].

The relative force of different ligands has been compared through many years of empirical work. The following list includes the main ligands [15]. **They are ordered by increasing force from left to right:**

I^-, Br^-, SCN^-, S^{2-}, SCN^-, Cl^-, NO_3^-, F^-, OH^- = $RCOO^-$, H_2O = RS^-, NH_3 = Imidazole, 2, 2' Bipyridine, Phenolate, NO_2^-, CN^- y CO.

The ligands most commonly coordinated to Fe in protein complexes are: the thiolate of cysteine, the imidazol of hystidine, the carboxylates in aspartic and glutamic acids, the phenolate of tyrosine; and to name few of the less frequent coordinations: diether of methionine, the amino of lysine, the guanidine of arginine, and the amide groups of asparagine and glutamine [15].

Depending on the identity of ligands and the geometry of complexes, some of the **3d** orbitals of Fe have a closer approach to some ligands than others; this is what determines the changes in their energy

content. This energy difference among them has been named **ligand field splitting energy**, represented by "**D**" [16].

In biological systems, metallic ions are frequently bound in a symmetric ligand environment; the order of orbitals filling varies depending on the relative magnitudes of "D" and on the energy involved in electron pairing ("P"); that means, electrons will finally place in such a way that their energy content will be the minimum.

In certain cases, the effect of approaching ligands to Fe in a given geometry generates a "**D**" so high, that makes more difficult to electrons to go into the highest energy **3d** orbitals, so they go to a more stable condition, moving in lower energy **3d** orbitals, despite the mutual repulsion they experience when they coexist with another electron in the same orbital.

In other cases, the "**D**" value is lower, in that condition the energy cost of having electrons in the higher energy **3d** orbitals is inferior than moving them into the lowest energy orbitals **3d**, next to an electron that repels it, and consequently setting an electron in each of the five orbitals, and just one of these will be filled with the 6th electron, generating a high-spin arrangement (with 4 parallel-spin electrons).

For a better understanding of the influence of the approximation of the ligands to the energy content of the electron orbitals "d", it can be considered the octahedral geometry around a metal ion (the most common of the molecular geometries).

The metallic ion can be pictured in the centre of a cube, which is crossed in each of its six faces by an axis of a Cartesian coordinate system (x, y, z). There are two orbitals within the five **3d** orbitals of Fe ions, named $\mathbf{d_{x^2-y^2}}$ and $\mathbf{d_{z^2}}$; both of them present their higher density and are located on the Cartesian axes. If a ligand approaches to the center of each of the six cube faces, the electrons in these **d** orbitals will experience the repulsion of ligand electrons. Unlike if the electrons would occupy the other **3d** orbitals ($\mathbf{d_{x^2-y^2}}$ and $\mathbf{d_z2}$).

In short, an electron requires different energy amount either to remain spinning in the same place or to move to any of the other three 3d Fe orbitals (which are farther from the influence of the ligands). The influence of the electric field of the ligand leads to a differentiation among orbital energy "**d**" (not previously observed), the aforementioned ligand field splitting energy "**D**". Strong ligands drive to a great energy difference between electrons $\mathbf{d_{z^2}}$ and $\mathbf{d_{x^2-y^2}}$ (with higher energy) and the remaining three **3d** orbitals (which contain less energy). The first referred ligands have orbitals with higher density of negative charge; weak ligands in contrast, propel to a smaller energy difference splitting, because they have less compact orbitals and their density of charge is comparatively low.

The oxidation state of ions also influences the differentiation of the energy content after splitting. As the oxidation state increases, the difference in energy content grows up.

The geometry of the ligands around the metal ion is also very important to ensure compliance with the described above, since the higher energy content in the orbital **2d**, owing to the negative electric charge repels other electrons moving towards on the orientation of the three Cartesian axes. In contrast, if the approximation of the electron clouds of the ligands is oriented in between axes, the closest proximity will be with the electrons of orbitals $\mathbf{d_{x-y}}$, $\mathbf{d_{x-z}}$ and $\mathbf{d_{y-z}}$ thus it would be these electrons which would require more energy to keep moving around. The electrons of the other two **3d** orbitals contain less energy; 4 electrons fill up the Fe orbitals, mutually neutralizing their spins, and the 2 remaining electrons move into two of the three highest energy **3d** orbitals.

The electrons of the two **d** orbitals located on Cartesian axes, in an octahedral geometry, can form **σ** bonds, reaching important electronic densities along the bonding axes between two atoms. Depending on how many ligands and from where they approach, these two orbitals can incorporate electrons since they are empty, due to their greater energy axis demand compared to the other three **3d** orbitals ($\mathbf{d_{xy}}$, $\mathbf{d_{xz}}$ and $\mathbf{d_{yz}}$). These last orbitals can form **π** bonds, which density along the bonding equal to zero.

In Figure 4, five **d** orbitals are represented, two of them with high energy, five electrons have been set, all with parallel spin, because in this case the filling of higher energy spaces predominates, in favor of releasing the repulsion between the two electrons occupying the same orbital. **Given the characteristics of orbitals here represented, the sixth electron will occupy any of the three orbitals with less energy. If those five electrons would have set in the three 3d orbitals with less energy, the sixth electron would fit in the remaining non-full-saturated orbital (among them), neutralizing in this way each spin and reaching a low-spin arrangement.**

8. How do ligands bind ionic iron?

The molecular orbitals are formed by the interaction of the electrons moving in atomic orbitals, and are able to do so in two ways: **constructive or destructive**, depending on whether their wave functions are added or subtracted in the overlap region.

Constructive interactions give rise to bonding molecular orbitals that only are formed when there are compatible symmetries; in such a way that the overlap of these causes an increase in electron density between atoms that determine a covalent bond.

Molecular orbitals can be **σ**, which are characterized by a high electron density on the axis between the two bonded atoms; they may also be **π** that results from the lateral interaction between atomic orbitals, to form a molecular orbital which reaches the greater electron density above and below the level located in the line that connects to the bonded atoms).

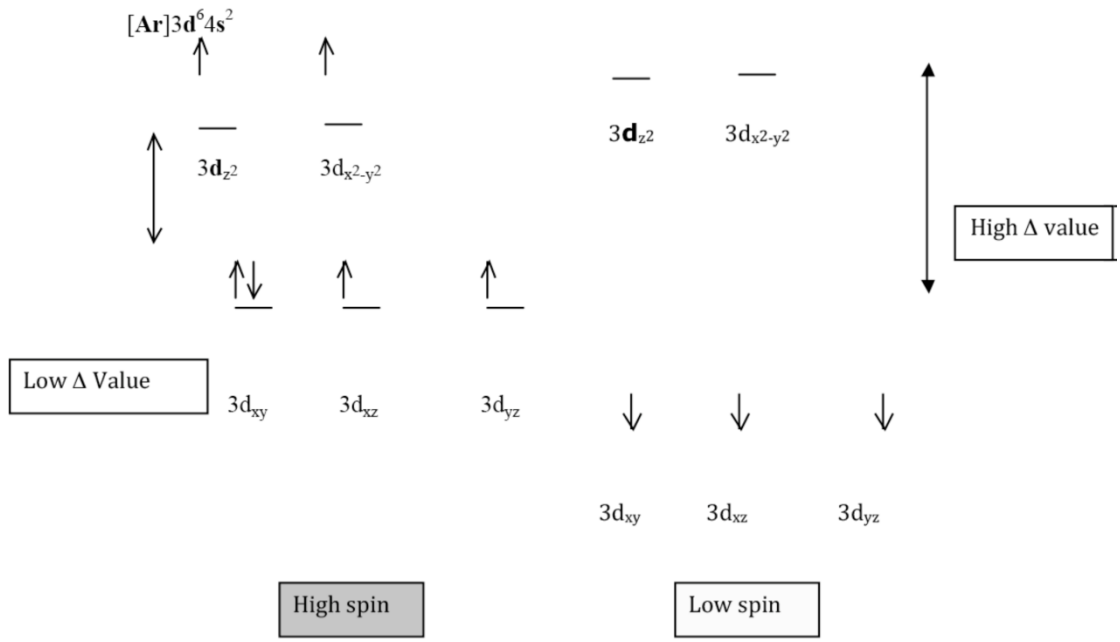


Figure 4. IN THE FIRST EXAMPLE HIGH-SPIN Fe^{+2} IS REPRESENTED, IN THE SECOND ONE THE ARRANGEMENT SHOWN CORRESPONDS TO LOW-SPIN Fe^{+2}.

Destructive interactions give rise to the anti-bonding orbitals (named σ^* and π^*). This kind of interaction happens when symmetries are not compatible, the wave-like movement of electrons leads to interference which ultimately decrease the density of the electron cloud in space among atoms [15].

The σ bonding molecular orbitals are formed from any atomic orbital full of electrons donated by the ligand to any empty orbital of the metallic ion. The π bonding molecular orbitals are formed with any donation of electronic density from the metallic ion to any π orbital of the ligand, or also from the π orbitals of the ligand to the d orbitals of the metallic ion. Molecular orbitals will have a greater similarity with those atomic orbitals that give rise to them and those that are closer in their energy content.

π bonds are generally weaker than σ. If we consider a coordination number of 6, two orbitals can be formed (one bonding orbital and one antibonding orbital) by each ligand; the metal also contributes with six orbitals (three **4p** orbitals, one **4s** orbital and two **3d** orbitals) giving together (ligands and metal) a total of twelve orbitals. In an octahedral geometry, the orbitals t_{2g} of the metal are more appropriate for the interaction, because they are already oriented on the Cartesian axes *x*, *y* and *z*.

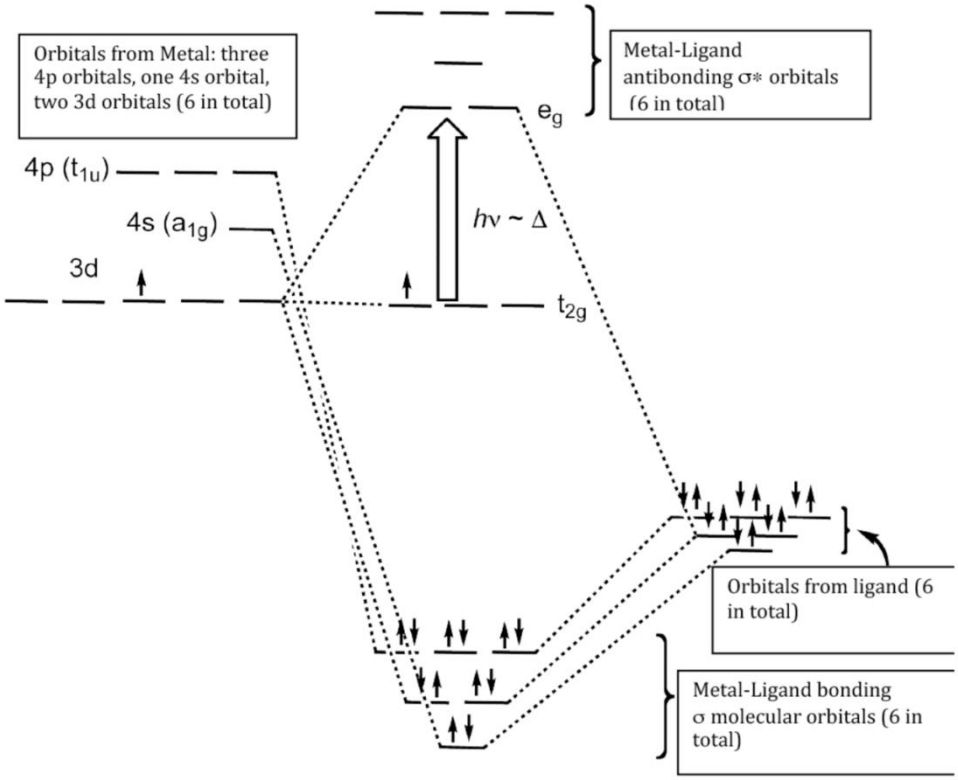


Figure 5. ENERGY OF ATOMIC AND MOLECULAR ORBITALS.

There are two cases to be considered with respect to this interaction: the orbitals t_{2g} are empty and the orbitals of the ligand are full and have less energy than electrons t_{2g}. In this case, there is a decrease in the magnitude of **D**, the electron density will be transferred from the ligand to metal, and the ligand is now acting as a π donator.

In the second case, the electrons of t_{2g}, which are the most appropriate to form π orbitals in a ML6 complex, transfer electron density to empty π orbitals of the ligand, which possess higher energy than t_{2g} orbitals of the metal, causing an increase in the value of **D.**

This explains the position of the spectrophotometric series of CN^- and CO, because they have π empty orbitals. The additional π bond is responsible of stabilizing the low oxidation states in the metal, allowing the delocalization of electrons from higher charge density caused by strong ligands binding, and releasing the charge in this way, then the complex stabilizes. In contrast, weak ligands like F^- or OH^- have π symmetry orbitals full of electrons, which act as donors and stabilize the highest oxidation state of the metal [15].

9. **What determines the lesser atomic radius of iron? How does it affect its behavior?**

Fe(II) has three possible radii: 0.77, 0.97 or 0.75 Angstroms, according to whether its coordination number is 4 (it is defined by the number of ligands surrounding the ion) and has high spin; if its coordination number is 6 with high spin; or if its coordination number is 6 with low spin, respectively. Almost all cations form complexes with coordination number 6. **Fe(III) has a ionic radius 22% lower than the Fe(II).**

Among transition metals, those of the period IV are small, of which Fe and Mn have the smallest radius. **The size of an atom or a ion is defined by the position of their outermost electrons**. This depends on two factors: 1) How many electrons form part of the atom or ion, which determines the outermost electron level. 2) How many positive and negative charges participate in the atom or ion to bring the most distant electrons closer to the nucleus, given the mutual attraction among them.

Table 3. ATOMIC RADII ARE ILLUSTRATED IN PICOMETERS, THE NUMBER OF OPTIONS THAT EXIST FOR THE FILLING OF THE "d" SUBSHELL AND ABUNDANCE IN THE EARTH'S CRUST OF TEN ELEMENTS OF TRANSITION ARE ALSO INCLUDED.

It can be seen that only 4 of them have two options for filling the "d" orbitals with electrons.

Transition Element	Metallic atomic radius (pm)	Number of options for the filling of "d" orbitals	Abundance (%)
Scandium	161	1	0.0025
Titanium	145	1	0.63
Vanadium	132	1	0.014
Chromium	125	2	0.0122
Manganese	124	2	0.11
Iron	124	2	6.2
Cobalt	125	2	0.003
Niquel	125	1	0.01
Copper	128	1	0.068
Zinc	133	1	0.012

The outermost subshell with electrons in Fe is the fourth, in contrast with other transition elements that have layers 5 or 6 occupied with electrons. Only Mn has a better arrangement of the **d** electrons than Fe, considering that none of its "**d**-orbitals" is filled with its 25 electrons; leading to an increase in volume, and avoiding this way, a greater repulsion that occurs when these orbitals are full.

Fe contains 26 protons in the nucleus, and one more electron than Mn, it has a greater force of attraction. Fe compensates the effect of repulsion, when one of the **d**-orbitals is full, both elements get the same radio. In Table 3 are shown in detail the atomic radii of the first series of transition elements; it also includes the abundance in the Earth's crust of each one of them and two ways to fill the **d** orbitals.

10. What is the availability of Fe? What state is the most common and which the most available?

We have already mentioned that Fe is very abundant in the Earth's crust, but is very scarce in aqueous solution (seawater contains 3 x 10^{-3} mg/l of iron). **Both the ferric and ferrous ions are capable of forming soluble hexahydrated complexes $[Fe(H_2O)_6]^{+2}$ and $[Fe(H_2O)_6]^{+3}$.** These complexes in aqueous solution exposed to clash with other water molecules, cause dissociation of protons from the complex (which is called hydrolysis).

The pKa of ferrous and ferric hexahydrates has very different values [12]. **The pKa of the ferrous ion is close to 7** (which corresponds to a Ka of 1 x 10^{-7}), **that is to say, one proton out of 10 million complexes is dissociated. In the case of Fe^{+3} the pKa value is between 2 and 3 (one proton in a number between 100 and 1000 complexes is dissociated).** This dissociation is very important, because when it happens, polymers of oxyhydrates and insoluble oxides are constituted. **For this reason, the ferrous ion (oxidation number +2), which hydrolyzes to a much lesser extent, is much more soluble in water than the ferric ion (oxidation number +3)**; ferrous ion has a solubility product close to 1 x 10^{-17} and its maximum solubility is about 1 mM at pH 7 (at this pH, the square of OH^- ion concentration is equal to 1 x 10^{-14} which multiplied by 1x10^{-3} of Fe^{+2} leads to the value of its solubility product).

In contrast, the Fe^{+3} is almost insoluble in water when it is present as trihydrate hydroxide, resulting from a dissociation of 3 protons of hexahydrate $[Fe(H_2O)_3(OH^-)_3]^{+2}$. Its solubility product is 10^{-39} at pH 7 (because at pH 7, there is a 10^{-7} M concentration of OH^- that raised the second power equals 10^{-21}) and reaches a dissolved concentration of 10^{-18} molar (whereas one mole contains on the order of 10^{23} ions, in 1ml of water with iron 10^{-18} M we would find about 100 **Fe^{+3}** ions) which is almost nothing, **while Fe^{+2} would be about 2 x 10^{17} ions.**

It is important to note that there are ways of the **Fe^{+3}** with less tendency to polymerize, as happens in the complex $[Fe(H_2O)_4(OH^-)_2]^{+3}$ whose dissolved concentration comes to be of the order of 10^{-9} M [8,5]. You can see that the decoupling of the ferric hexahydrate complex predominates at pH values above their pKa. Also it should be noted that the trihydrated hydroxide is favored with the maximum concentration of OH^- in high pH.

Although the dominance of the Fe(II) would be expected by its greater solubility in intra- and extra-cellular environments, the incorporation of iron in biological complexes (structure synthesized by living organisms to capture iron) favors the ferric ion, given its smaller ionic radius and greater charge density.

Normally, the Ka towards Fe(III) is higher than for Fe(II), for example in transferrin and lactoferrin is 10^{20} for Fe(III), and only 10^{3} for Fe(II); it also facilitates the self-oxidation from Fe(II) to Fe(III), as it occurs inside the ferritin which contains thousands of Fe(III) ions [17,18].

In addition to these conditions, it may be considered that **at physiological concentrations of oxygen, Fe^{+3} is the most stable state of Fe in the majority of biological complexes.** In these circumstances, **living organisms have developed a set of biomolecules capable of coupling iron by reducing the polynuclear complexes, while they get the ability to keep in solution each compartment, and providing elements strategically located, for transforming it in Fe^{+2}, for short periods of time.** Therefore, its transfer is facilitated because of its higher solubility, **after that, Fe is reconverted to Fe^{+3}, reducing to the minimum, the risk of increasing reactive toxic radicals.**

Therefore, reduction reactions play a critical role in Fe metabolism, due to its transmembrane transport, incorporation of storage proteins and the synthesis of heme group take place with Fe(II) [8]. On the other hand, Fe that constitutes hemoglobin predominates in ferric form when hemoglobin is oxygenated, while deoxyhemoglobin contains preferably Fe(II).

It is important to consider that the reduction of the dioxygen molecule with Fe^{+2} results in the formation of superoxide, that leads to the well known Haber-Weiss-Fenton sequence, generating hydroxyl radicals. Thus, **it is very important to maintain sufficiently oxidized conditions in order to reduce to the minimum the Fe(II) participation in processes that put at risk cellular functions,** although there is evidence that suggests the competent catalytic participation of ferrous heme proteins and peroxides, that could involve routes that do not generate hydroxyl radicals [19].

The reduction promoted by Fe^{+2}, the high solubility of this ion, and its involvement in the generation of free radicals, let us appreciate the reason why Fe has toxic effects when exceeds normal levels. In human beings, about 25 mg of Fe are required daily for erythropoiesis; the main part comes from retrieving Fe already containing cells and only 1 - 2 mg come from the intestinal absorption).

In counterpart, its participation in processes of energy production and oxygen transport, determines that Fe deficit leads to cell damage, reduction of growth, proliferation, hypoxia and death.

Due to the duality of Fe (essential and toxic), evolution of organisms led to the establishment of elaborated systems that assure Fe availability as it is needed, and prevent its toxic overabundance [6].

Aqueous systems inevitably have a certain concentration of OH^- which at pH 7 is 10^{-7} M. **Le Chatelier's Principle** establishes that when an equilibrium system is disturbed, it moves in such direction to reduce the disturbance. In consequence, increasing hydroxyl ions will drive the reaction towards ferric hydroxide formation, which after forming aggregates will precipitate, leaving Fe^{+3} even less solved.

When the Earth´s atmosphere was reducing, Fe(II) predominated, which aqueous solution concentration was enough to allow an adequate supply to cells. **However, when oxygen predominated in the atmosphere, the dominance of Fe(III) against Fe(II) was promoted,** and given its low solubility and the abundance of oxygen, it forms huge deposits of insoluble Fe oxide, causing a lack of Fe availability suitable for living organisms [20].

Natural selection favored those organisms that could remove Fe(III) from the deposits, by secreting peptide derivatives like **siderophores** or **ferrichrome**. These biomolecules, due to their high bond strength, are **able to remove Fe^{+3} from Pyrex glass** [21].

When free Fe^{+3} exceeds solubility levels, it precipitates. **One way to increase dissolved Fe is turning ferric into ferrous ions, because Fe^{+2} is much more soluble; however, the extracellular environment conditions of the most aerobic cells, cause the oxidation of ferrous into ferric ions.** The same occurs in the Earth´s crust, since despite being the second most abundant metal, Fe^{+3} is not available because it is forming oxyhydroxide polymers.

Proteins or complexes of low molecular weight that associate ferrous or ferric ions, allow associating Fe ions bound to the polymer, thanks to the high affinity among them, to the extent of reaching values of $\mathbf{10^{53}}$, **as occurs in some siderophores.** But such a high affinity prevents the captured Fe^{+3} for being released. At some time it was thought that the way to solve this problem was digesting the protein component linked to such a high affinity to Fe, but according to accumulated evidence, **the mechanism is to reduce Fe^{+3} to Fe^{+2} inside the complex**. **Given the much lower affinity of the ferrous ion into the complex, with an affinity constant close to 10^8, once reduced, it can be dissociated, leaving the Fe at the disposal of the cell.**

Fe(II) is self-oxidized at pH function's speed [22]. This behavior is due to a simplified speed equation, in which the velocity is equal to a factor multiplied by $[Fe(II)][O_2][OH]^2$ [18]. It should not be forgotten that **there is a balance between Fe^{+2} and Fe^{+3}; when increasing the OH^-, the precipitation of Fe^{+3} get promoted, and when its concentration decreases, oxidation of Fe^{+2} is favored. According to the Law of Mass Action, the previously excluded ferric ion would be replaced in the solution, and on the other hand, O_2 will directly oxidize Fe^{+2}.**

11. How is the ionic radius of Fe, its solubility and affinity for ligands related?

In spite of having an excess of three positive charges with respect to their electrons, Fe III has the same charges in a smaller volume than Fe II (which has an excess of two positive charges in a larger volume),

thus having a greater concentration of positive charge, has a strong tendency to capture anions. In this way, if the ion comes in contact with water, and given that there is a concentration of H_3O^+ ions and OH^- anions whose product invariably turns out to be 10^{-14}, the only way to substantially reduce the formation of ions OH^-, that are responsible for precipitating the Fe^{3+}, is raising the concentration of H_3O^+, to what we call acidify the medium.

Reducing the concentration of OH^- is important because the aqua-ionic complexes of the Fe^{+3} become polynuclear structures that have a $Fe_pO_r(OH)_s^{3p-(2r+s)}$ composition, which are polyelectrolytes with positive charge; as colloids, they can be coagulated with specific adsorbents anions. In these polynuclear structures, the peripheral Fe atoms are coordinated with at least one molecule of water, which if gets deprotonated by pH increase can also cause coagulation [5]. In counterpart, the Fe^{+2} with its largest ionic radius, presents a lower density of electric charge (two positive charges in greater volume), is attracted with less intensity and therefore it is released from hydroxide complexes much more easily in order to achieve a concentration up to 0.2 mM in its free form.

12. Final considerations

As already mentioned, living organisms evolved in such a way that they have mechanisms to take Fe from the environment, although it is abundant, it is not available in the dissolved form after the establishment of an atmosphere with O_2.

This developed mechanism consists in the construction of biomolecules with high affinity for the Fe^{+3}, the provision of components that reduce it to Fe^{+2}, which is more than 1 billion times more soluble than the ferric ion at neutral pH, and which in many cases is the way to incorporate complexes or structures that transport it. Generally, inside these structures it becomes ferric ion, which affinity constant is much higher in such an extent, that to release Fe from the complex, works in two directions: reduce it and decrease the pH, since acidification, disturbs electronic pairs of ligands that interact with Fe, without prevailing in its reduced form, given the danger associated with its ability to produce free radicals by interaction with oxygen and water.

Although the course of evolution pointed to take advantage of that with slight adjustments in the electronic structure of Fe, these can cause changes in affinities, that are also influenced by the geometry. This option incorporates elements of regulation including modulation of the activity directly involving the Fe (such as the transport of O_2 in hemoglobin, the catalytic activity of enzymes that require Fe in its active site), or also in an indirect way, as occurs in proteins with Fe-sulfur centers. In this respect, evidence supports the participation of these centers in the regulation of the transcription and translation, having variants with the capability to respond to every environmental stimulus and use of different mechanisms to deliver answers, including the assemble of clusters, its conversion or redox self-regulation [23], which may constitute the ways of control of proteolysis, that recently have been considered [24].

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Chapter 2

Metabolism of Iron in Humans

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Abstract

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Abstract

Iron is an essential element for microbes, plants and higher animals. The primary role of iron in mammals is to provide a binding site for oxygen in the heme moiety of hemoglobin. More than two thirds of the iron content in the body is incorporated into hemoglobin in developing erythroid precursors and mature red cells. Most of the remaining body iron is found in hepatocytes and reticuloendothelial macrophages, which serve as storage depots. Body iron metabolism includes iron supply through food, nutrients that favor or inhibit iron absorption, intestinal epithelium integrity, the quantity of circulating transferrin, and the status of the storage depots at any given time.

Iron homeostasis is mainly achieved by controlling its absorption, rather than its excretion. Absorption increases during iron depletion and decreases during iron overload. In humans, recycling the iron from hemoglobin of senescent red blood cells strictly preserves iron. Macrophages in the spleen phagocytose erythrocytes, metabolize hemoglobin and iron is bound to transferrin for reuse.

Humans have no a physiological pathway for iron excretion, thus balance is attained by intestinal absorption. Hepcidin is a liver-produced peptide hormone and is thought to be the central regulator of body iron metabolism. It controls extracellular iron by regulating its intestinal absorption, placental transport, recycling by macrophages, and release from the stores. Hepcidin inhibits the cellular efflux of iron by binding to ferroportin and inducing its degradation.

Ferritin and hemosiderin are the most important iron storage proteins in humans. Serum ferritin levels are a reflection of body iron stores in the absence of pathological conditions, which raise ferritin levels, a known acute reaction protein.

Iron deficiency is a global public health problem with unique cultural, dietary, and infectious factors that are difficult to overcome. Even in developed countries, 20% to 30% of infants are iron deficient by 1 to 2 years of age and 4% to 12% are anemic. Iron deficiency is the most common cause of anemia in developing countries. It is clinically occult in its early stages and usually not diagnosed until the development of anemia. The most troublesome consequence of iron deficiency may be its long-term neurocognitive deficits. Because of their rapid growth, children under three years of age are the most prone to develop iron deficiency. Prevention of iron deficiency must be accomplished through parental education and dietary iron supplementation, particularly in infants at this age**.**

Hereditary hemochromatosis consists of a group of genetic alterations that result in a lack of regulation of iron absorption from dietary sources, resulting in an excess of deposition in tissues, such as liver, endocrine glands, heart and skin.

Keywords: ferritin, ferroportin, hemosiderin, hepcidin, iron, iron deficiency anemia, iron overload, transferrin.

1. Introduction

Iron (Fe) is an **essential metal** in the biological processes of many organisms, particularly those in an oxygen-rich environment. The principal role of iron in mammals is to **provide a binding site for oxygen in the Heme moiety of Hemoglobin**. Iron has the capacity to accept and donate electrons readily, converting ferrous (**Fe^{++}**) into ferric iron (**Fe^{+++}**). This capability allows it to participate as an important component in the **enzymatic transfer of electrons** in reduction/oxidation reactions that are performed by **cytochromes**, **peroxidases**, **ribonucleotide reductases**, and **catalases** [1].

This reactivity, which makes iron essential, **also has the potential to cause significant damage to biological systems, if it is not attenuated by iron-binding proteins**. If the iron-binding capacity is exceeded, **free iron is capable of promoting the formation of free radicals** that are described by **Fenton's reaction**. In this reaction, hydrogen peroxide (**H_2O_2**) is transformed into **hydroxyl radicals (OH)**. This radical causes cell damage in the DNA, proteins and cell membrane [1,2,3].

Aerobic cells have developed diverse mechanisms to protect themselves from these lethal reactions by carrying iron ions bound to the transport protein –**Transferrin**- and storing them as **Ferritin** and **Hemosiderin** inside cells. Under normal conditions, free iron circulates in trace amounts.

Body iron metabolism is based on a highly efficient system of iron **conservation and recycling**. Under normal physiological conditions, **iron enters the human body through carefully regulated intestinal absorption and is lost only through menstrual bleeding and exfoliation of skin and mucosal cells**. There is no organ physiologically capable of iron elimination; therefore absorption is the only way to regulate iron storages in the body. **Iron cellular metabolism involves absorption, regulation and its utilization in cellular processes.**

2. Iron distribution in the body

Adult men have approximately 40 to 50 mg of iron per kilogram of body weight. Premenopausal women have lower iron stores as a result of their recurrent blood loss through menstruation. Approximately **two thirds of the body's iron content (30 mg/kg of weight) is incorporated into Hemoglobin in developing erythroid precursors and *mature red cells*** and 6 to 7 mg/Kg are found as **Myoglobin** in tissues. A small fraction, less than 0.5% of the total corporal iron, is found in **Transferrin (Tf)** in plasma and in the extracellular tissues.

Even smaller quantities of iron are found in many heme-type enzymes (**C*ytochromes, Catalases, Peroxidases***) and in non-heme enzymes (***Ribonucleotide-Reductases, Metaflavoproteins, Sulfur-iron proteins***). Remaining body iron, 5 to 6 mg/kg in women and 10 to 12 mg/kg in men, is stored as **Ferritin** and **Hemosiderin** mainly in the hepatocytes and macrophages of the liver, spleen, bone marrow and muscle [3,4,5]. Distribution of iron in the adult body is shown in **Figure 1.**

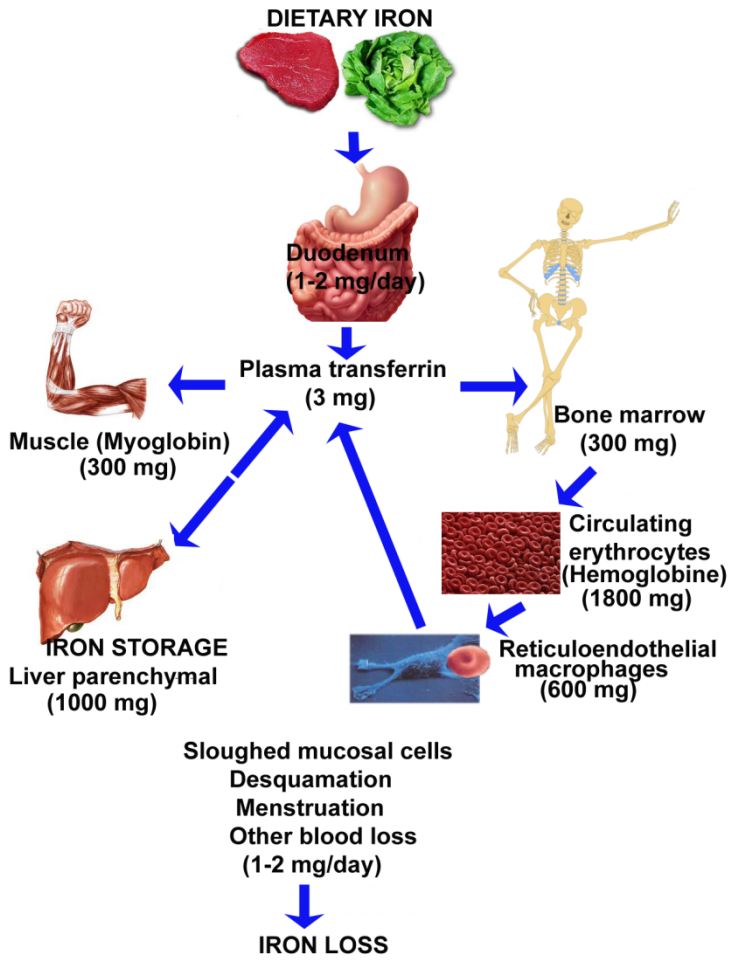

FIGURE 1. IRON DISTRIBUTION IN HUMAN BODY.

1-2 mg of iron is absorbed and lost every day. Dietary iron is absorbed by duodenal enterocytes. Iron circulates in the plasma bound to Transferrin. Most of the iron in the body is incorporated into hemoglobin in erythroid precursors and mature red cells. Approximately 10-15% percent is present in muscle fibers (in Myoglobin) and other tissues (in enzymes and cytochromes). Iron is stored in parenchymal cells of the liver and reticuloendothelial macrophages. These macrophages provide most of the usable iron by degrading Hemoglobin in senescent erythrocytes.

3. Iron absorption

The daily average requirement to support erythropoiesis is 20 mg to 25 mg; however, only 1 to 2 mg of this is absorbed from the diet. Most of the daily requirement is supplied by **recovery of erythroid iron through phagocytosis of senescent erythrocytes by the reticuloendothelial macrophages and degradation of Hemoglobin**. This **recycled iron is then made available to the developing erythroid**

precursors in the bone marrow. Smaller amounts are also recycled from myoglobin and some redox enzymes.

Daily iron loss is approximately 1 to 2 mg and it must be replaced through iron absorption from food by duodenal enterocytes. Absorption takes place in the **presence of acid pH,** mainly in the **duodenum** and the **proximal jejunum**, and less so in the distal segments of the small intestine. This transition may be explained best by the **quantity of receptors at the brush border of the proximal small intestine**. Cells that are located at the bottom of the crypt mature on their way out to the brush border. When they reach the top they part to the intestinal lumen and the intracellular iron is eliminated. This maturation process lasts from 3 to 5 days [4].

Acid production in the stomach serves to lower the pH in the duodenum, enhancing the solubility and the uptake of ferric iron. Some dietary factors influence iron absorption: **ascorbate and citrate increase iron uptake acting as weak chelators** to help to the metal solubilization in the duodenum and Transferrin it readily to the mucosal cells. Conversely**, plant phytates, tannins, phospho-proteins and calcium inhibit iron absorption.** These compounds chelate iron and prevent its uptake. **Normally only about 10% of the dietary iron is absorbed and this value increases with iron deficiency** [6].

Dietary iron is available in two forms, the ferric form (Fe^{+++}) or as heme iron, the ferrous form (Fe^{++}). In the intestinal lumen iron is in its ferric form**, and to be absorbed it is reduced to its ferrous form by an enzyme present in duodenal cells, the Duodenal Cytochrome-b Reductase (Dcytb)**. Once in the ferrous state, **Fe^{++} is transported in the enterocyte to its basolateral border, by the Divalent Metal Transporter-1 (DMT1)**, an energy-dependent symporter that also transports diverse metals (manganese, cobalt, copper, zinc, cadmium and lead)

Another source of dietary iron is heme iron, a molecule that consists of a **protoporphyrin ring** that binds iron. **Heme results from the breakdown of Hemoglobin and Myoglobin found in meat products, and intestinal cells through its union with the Heme Carrier Protein-1 (HCP1) absorb it. HCP1 is expressed at high levels in the duodenum** and, upon binding of heme to HCP1 on the cell surface the complex is internalized to the cell by receptor-mediated endocytosis. The resultant endosomal vesicles migrate to the endoplasmic reticulum, where **iron is released from Heme by the Heme Oxygenase-1 (HO1)** that is found on the reticulum surface; **iron released from Heme is imported into the enterocyte by DMT1.** Heme iron is absorbed easily and it is not affected by dietary composition.

Inside the enterocyte, iron has two possible fates: (1) It may be stored as Ferritin or (2) it may be transferred across the basolateral membrane to reach the plasma. These two pathways are not mutually exclusive. Iron that remains in the form of Ferritin will be eliminated when the enterocyte completes its life cycle and parts to the intestinal lumen. Iron that is transferred into the basolateral membrane is then exported through it by an **exporter protein: ferroportin (FPN1).** Iron in its ferrous state has to be oxidized by **Ferroxidase Hephaestin (Hp)** in the intestinal cells and by ***Ceruloplasmin*** in non-intestinal cells. Iron is

then bound to Transferrin and carried by plasma to the cells requiring iron [2,3]. **Figure 2** describes iron absorption in the enterocyte.

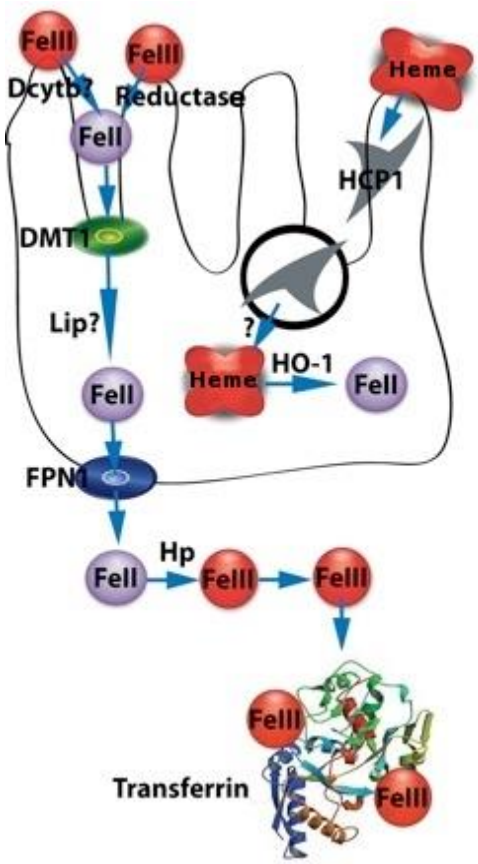

Figure 2. IRON ABSORPTION IN THE ENTEROCYTE

Non-heme dietary iron is taken up in the enterocytes of the duodenum after Fe (III) is reduced to Fe (II) by the enzyme: Duodenal Cytochrome-b Reductase (Dcytb). Fe (II) is transported into the cytosol by Divalent Metal Transporter-1 (DMT1). Heme iron is transported across the membrane by the Heme Carrier Protein-1 (HCP1). In the enterocyte Fe (II) can enter the cytosolic compartment, the Labile Iron Pool (LIP) or been exported through the basolateral membrane of the enterocyte by Ferroportin-1 (FPN1). Fe (II) is oxidized by Hephaestin (Hp) to Fe (III) before it is bound to Transferrin (Tf) and transported by the circulation.

Ferroportin is the only known mechanism of iron efflux from the enterocyte; it is also present in macrophages and hepatocytes. In turn, **the hormone Hepcidin negatively regulates FPN.** Hepcidin binds directly to ferroportin introducing it to the cell to be degraded. Lack of FPN in the cellular membrane blocks iron exit from the cell. This mechanism is sufficient to explain the regulation of iron absorption, because absorptive enterocytes only perform their function for two days before being shed from the tip of the villi into the intestinal lumen.

Iron transportation by FPN across the basolateral membrane determines whether iron binds to Tf and is delivered to plasma or removed from the body with shed enterocytes. When iron stores are adequate or high, liver produces hepcidin, which circulates to the small intestine. There, hepcidin causes FPN to be internalized, blocking the sole pathway for the transfer of iron from enterocytes to plasma. When iron stores are low, hepcidin production is suppressed, and FPN molecules are displayed on basolateral membranes of enterocytes, and there they transport iron from the enterocyte cytoplasm to plasma Tf [7]. **Figure 3** shows a diagram of the mechanism of action of hepcidin.

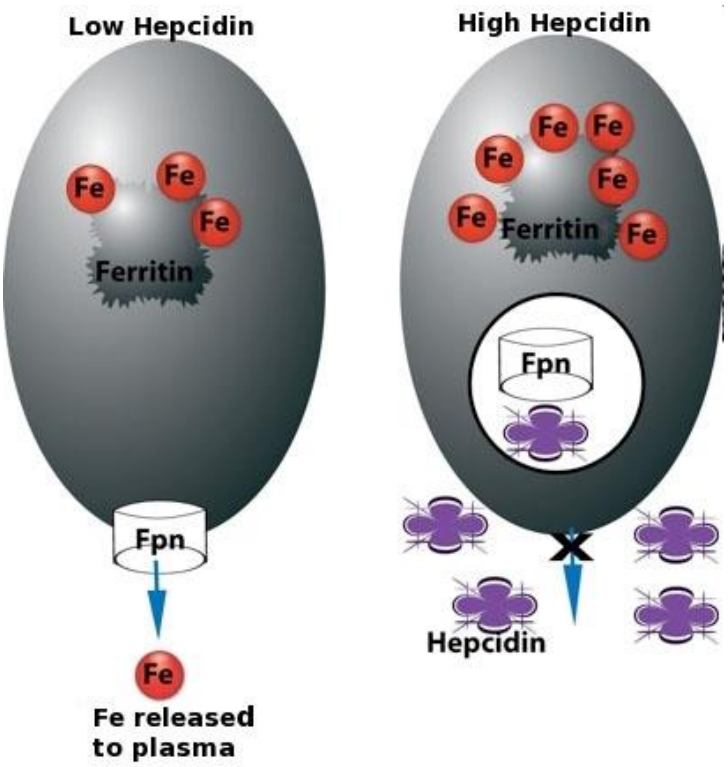

Figure 3. MECHANISMS OF ACTION OF HEPCIDIN.

Hepcidin regulates Ferroportin (FPN) expression at the basolateral membrane of enterocytes. Liver secretes Hepcidin, which interacts with FPN molecules at the basolateral membrane, leading to endocytosis and degradation of FPN. Iron export from enterocytes is decreased.

Similarly, the Hepcidin-FPN interaction also explains how macrophage recycling of iron is regulated and accounts for the characteristic finding of iron-containing macrophages in inflammatory states characterized by high production of Hepcidin. **The regulation of body iron is strictly controlled by Hepcidin hormone.** Hepcidin is **a negative regulator of iron transport in the small intestine and**

placenta; it induces **iron retention** in **spleen** macrophages engaged in the recycling of iron from senescent erythrocytes, as well as the **exportation of iron** from hepatocytes.

Liver production of Hepcidin is regulated by: a) body iron stores, b) inflammation, c) erythropoietic demand and d) hypoxia, through regulation pathways as the expression of **HFE genes** (hemochromatosis gene product), **TfR2** (**Transferrin receptor-2**) and **HJV** (**Hemojuvelin**). All these pathways interact with liver cells to initiate the production of sufficient hepcidin for correct maintenance of iron homeostasis.

When oxygen delivery is inadequate in tissues, the homeostatic response is to produce more erythrocytes, hepcidin levels decrease, its inhibitory effects on FPN diminish, and more iron is made available from the diet and from the storage pool in macrophages and hepatocytes. **Interlukin-6 (IL-6) is released by inflammation, which induces Hepcidin production;** this hormone increment is accompanied by a decrease in serum iron and in Tf saturation and **hypoferremia** [7,8].

4. Iron transport by Transferrin

Once iron is transported across the basolateral membrane of enterocytes, it is bound to Transferrin, the principal transport protein of iron in serum and extracellular liquid. It is a single chain glycoprotein containing two binding sites for iron. **It is synthesized in the liver** with a half-life of 8 days.

Under normal conditions, Tf iron-binding sites are approximately one third saturated and the unbound iron is virtually no existing; **the Tf-iron complex is taken to the liver and bone marrow to be incorporated to hemoglobin or to be stored.** The liver receives blood from the portal circulation. **On the surface of the cellular membrane, Tf binds to the Transferrin receptor (TfR). All nucleated cells have TfR.** With the exception of liver, the largest number of receptors is found in the placenta and in the developing red cells in the bone marrow.

Tf receptors have a high affinity for the iron-Tf complex; diferric Tf has a higher affinity than monoferric Tf or apoTf (without iron). **Once diferric Tf binds to its receptor, it is introduced into the cell through invagination of the membrane to form endosomes, covered with clathrin** (a polyhedric protein in the shape of a net covering the endosome). Inside the endosome, pH becomes acidified until pH is 5.5 through an **ATP-dependent proton pump.** At this pH Tf releases iron, which binds more firmly to the TfR.

Iron is transported out of the endosome in a process that involves DMT1 and an endosomic oxidoreductase which changes ferric iron bound to Tf into ferrous iron to be transported by DMT1 through the endosomic membrane. The TfR undergoes conformational changes that contribute to iron release from Tf. Transferrin, bound to the receptor, returns to the cell surface where they dissociate at neutral pH and become available for reuse. **Transferrin Cycle** is shown in **Figure 4.**

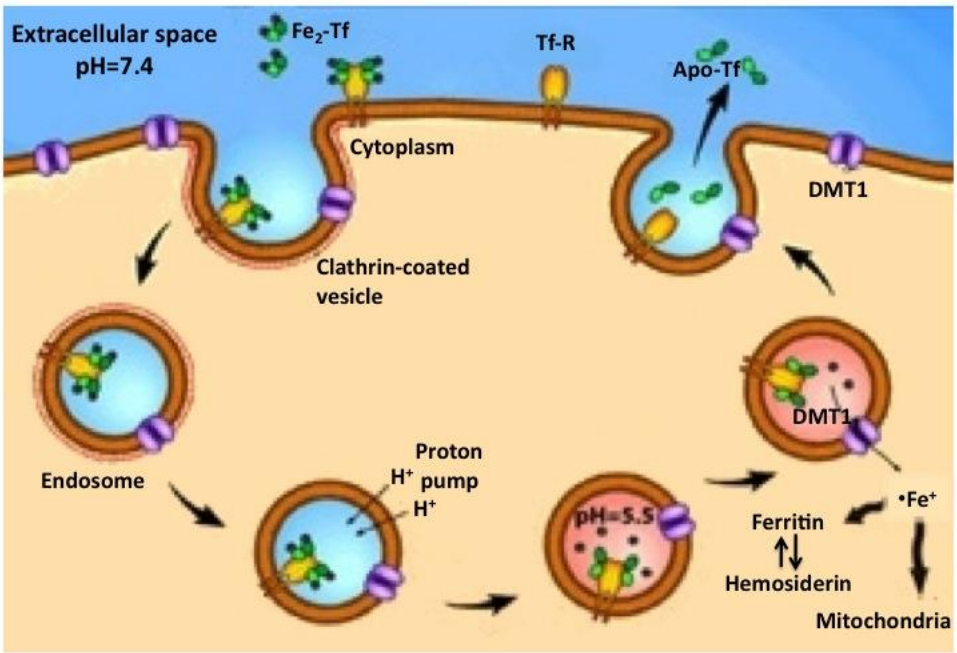

FIGURE 4. THE TRANSFERRIN CYCLE.

The two-iron Transferrin complex (Fe_2-Tf) binds to Transferrin receptors (TfR) on the surface of erythroid precursors. These complexes localize at clathrin-coated pits, which invaginate to form specialized endosomes. A proton pump decreases the pH within the endosomes to release iron from Tf. The iron transporter DMT1 moves iron across the endosomal membrane, to enter the cytoplasm. Transferrin without iron (ApoTf) and TfR are recycled to the cell surface, where each can be used for further cycles of iron binding and iron uptake. In erythroid cells, most iron moves into the mitochondria, where it is incorporated into protoporphyrin to form Heme. In non-erythroid cells, iron is stored as Ferritin and Hemosiderin.

5. Iron metabolism in mitochondria

After Transferrin has been released in the endosome, iron must cross the plasma membrane to enter the cytosol. **The mitochondrion is the site for Heme synthesis and the mayor site for iron-sulfur (Fe-S) cluster biosynthesis that are utilized as enzyme cofactors.**

Iron is transported from the cytosol to the mitochondrial membrane by a transporter identified in murine erythroblasts as **Mitoferrin;** it is used in many metabolic processes, specially Heme and Fe-S cluster synthesis. Iron binds to **protoporphyrin IX** to form Heme, which is then transported out of the mitochondria by proteins such as **cytochromes** to join globin chains and integrate the Hemoglobin. The transporter protein responsible for heme release remains unclear, and three molecules have been identified as **possible mitochondrial heme exporters:** the **Breast Cancer Resistance Protein (ABCG2)**, the **ABC-**

Mitochondrial Erythroid Transporter (ABC-me) and the **Feline Leukemia Virus Subgroup C Receptor (FLVCR).**

The molecular mechanism and contribution of these proteins to Heme transport in the mitochondria remains to be determined. It is hypothesized that **the mitochondrial protein Frataxin regulates mitochondrial iron utilization, acting as a metabolic switch between Heme synthesis and the genesis of [Fe-S] clusters**. **Inside the mitochondria, iron is stored as Ferritin.** [2]

6. Iron storage

Metabolically inactive iron is stored bound to Ferritin and Hemosiderin molecules, which represent 30% of total body iron. Ferritin is the main cell mechanism to store iron. Ferritin molecules are complex 24-subunit heteropolymers of H (heavy) and L (light) protein subunits. They assemble to form **multimeric complexes** with a central cavity in which up to several thousand-iron molecules can be stored. **The H Ferritin subunit has Ferroxidase activity** transforming ferric iron into ferrous iron, allowing its incorporation into Ferritin.

ApoFerritin (Ferritin without iron) forms a sphere of 6 to 8 nanometers with a central cavity in which up to 4500 iron atoms can be stored. Ferritin molecules aggregate into clusters that are digested and degraded by **lisosomes**. The end product of this process, **Hemosiderin, is an amorphous conglomerate of denatured protein and lipid interspersed with iron oxide molecules.** Iron trapped in this insoluble compound constitutes an end-stage product of cellular iron storage; it remains in equilibrium with soluble Ferritin. Ferritin, in turn, is in equilibrium with iron in complex with low molecular-weight carrier molecules.

The bioavailability of iron stored in Hemosiderin is much lower than that in Ferritin. Most of the Ferritin is located inside of cells and there is a measurable amount in serum. The origin of extracellular Ferritin is not clear. It is likely that some portion originates from necrotic cells but most of it seems to be secreted. **Circulating Ferritin appears to be formed exclusively by L chain subunits,** in contrast to intracellular Ferritin containing a mixture of H and L subunits. **Serum Ferritin levels decrease during iron deficit and increase in overload conditions.** In the absence of hepatic disease, infection or chronic inflammatory process, Ferritin levels reasonably reflect the total amount of stored iron in the body [6,9]

7. Iron deficiency

Iron deficiency is the most common micronutrient deficiency in developed and developing countries, particularly in children and premenopausal women.

7.1 Epidemiology

The most frequent iron deficiency illness is the **Ferropenic anemia.** This kind of anemia is the most frequent in the world and at all ages and it is a major public health problem in infants and young children, with an estimated prevalence of 3 to 80% in different populations [10]. It has been reported that 20% to 30% of infants by 1 to 2 years of age are iron deficient in developed countries and 4% to 12% are anemic [4].

In the last three decades, since the introduction of programs of iron fortification in food, such as milk and cereals, the promotion of breastfeeding, and introduction of cow milk in the diet at one year of age [11], there has been an important decrease in the incidence of this deficiency in developed countries [11]. **Iron deficiency is more common in developing countries** where children consume iron-poor food and are afflicted by infections such as **malaria and infested with hematophagous parasites (*Anchylostoma duodenale, Necator americanus and Trichuris trichiura*).** Halterman [12] reported iron deficiency in 3% of schoolchildren and adolescents between 6 to 16 years old and 8.7% in adolescent girls.

7.2 Natural history of iron deficiency

Iron deficiency anemia is developed in a gradual way. There are three identified stages of its evolution with specific alterations:

First stage: **it initiates with a decrease of iron intake or an increase in the requirements that leads to consumption of the stored iron**; the storages depletion causes **a decrease of Hemosiderin in the bone marrow, which is reflected in a reduction of serum Ferritin levels.**

Second stage: once iron stores are depleted, insufficient iron leads to **a decrease in the level of seric iron, and a drop in Transferrin saturation.**

Third stage: When the **sideroblast percentage** in the bone marrow is lower than 10%, Transferrin saturation is less than 16% and serum iron is < 60 μg/dl, **erythroblast production decreases, erythrocytes are produced in smaller size (microcytic) and Hemoglobin concentration in erythrocytes declines (hypochromic).** These changes result in a decrease in Hemoglobin levels and a **Microcytic Hypochromic Anemia** is established [5,13]

7.3 Etiology

Iron-Deficiency Anemia may be caused by:

A. Inadequate iron intake.

B. Absorption defect.

C. Chronic blood loss.

A. Inadequate iron intake

The human newborn has an iron stock of approximately 75 mg/kg at birth. Iron storage is determined by birth weight and hemoglobin concentration. **A minor iron deficiency in the mother generally does not affect iron supplied to the fetus during pregnancy, but a moderate or major deficiency may cause Hemoglobin decrease and low birth weight.**

The iron stock of a normal newborn is sufficient to ensure the production of Hemoglobin for the first 4 months of life. Subsequently, **iron supply is rapidly depleted when dietary iron is insufficient** and anemia may appear during the latter half of the first year. Premature babies, diabetic mothers' babies and low weight newborns have lower iron stocks than full term newborns and higher risk of developing anemia earlier in life.

Breastfed children have an adequate iron intake because iron has a high bioavailability. Children fed with industrialized milk have to receive fortified formulas containing from 4 to 12 mg of iron per liter to diminish the risk of developing anemia.

During development there are several stages of rapid growth in which iron requirements increases 1 to 2 fold:

In the first year of life, the newborn triplicates its weight, triplicates its blood volume and duplicates its body Hemoglobin; in premature babies growth is even faster. Requirements decrease during the second year because the growth speed decreases as well, but it increases during adolescence, a stage in which iron requirement is higher than the intake through diet and body storages. **Dietary iron intake must be 10 mg a day until the first 6 months of age and 15 mg from 6 months to 3 years of age considering that only 10% of the food iron is absorbed.**

In women, high requirements persist due to the loss of blood during menstruation. **Pregnancy results in a loss of blood equal to 1200 to 1500 ml.** Mother delivers an average of 270 mg of iron to the fetus; the umbilical cord and placenta have an extra 90 mg and an estimated 150 mg are lost during delivery and through lochia, reaching an approximate total loss of 500mg. During pregnancy, red blood cell mass increases more than a third which represents an additional requirement of 500 mg. The iron requirement during this stage increases to 30 mg per day in the diet.

Iron bioavailability in food may be low, moderate or high. (**Chart 1**) [14]. A diet based on cereals, roots, tuber plants and limited amount of meat, fish and ascorbic acid has a low availability; from this diet, 5% of iron is absorbed and supplies up to 0.7 mg of the metal per day, an inadequate amount to assure requirements. A moderate diet contains the above mentioned food and a regular amount of meat, fish and ascorbic acid; from this diet 10% of iron is absorbed and supplies approximately 1.4 mg of the ion, an adequate quantity for 50% of the women.

A high bioavailability diet of iron has generous quantities of red meat, poultry, fish and ascorbic acid; from this diet 15% of iron is absorbed and supplies approximately 2 mg of the ion, a quantity that is adequate for most individuals.

CHART 1. IRON BIOAVAILABILITY IN SEVERAL FOODS

	BIOAVAILABILITY OF IRON		
FOOD COMPONENTS	**LOW**	**MIDDLE**	**HIGH**
CEREALS	Corn, oat, rice, whole-wheat flour.	Corn flour. White flour	
FRUITS	Apple, avocado, grapes, banana, pear, peaches, strawberry.	Melon, mango, pineapple.	Lemon, orange, papaya, tomato, guava.
CROPS	Lentils, spinach.	Carrots, potatoes.	Broccoli, cabbage, squash, cauliflower, radish.
SEEDS	Almonds, coconut, Brazil nut, peanut, nut.		
HIGH PROTEIN FOOD	Egg, soy, soy flour		Fish, red meat, poultry.

Modified from [14].

B. Absorption defect

It is a seldom cause of deficiency generally observed after surgery such as **gastric or intestinal resection, or in patients with malabsorption syndromes, such as steatorrhea, celiac disease or sprue.**

C. Chronic blood loss

In men and postmenopausic women, iron deficit is most frequently caused by secondary gastrointestinal bleeding associated with conditions such as: **hyatal hernia, esophagic varices, duodenitis, peptic ulcer, hemorrhoids, etc.** Iron deficit may be the first sign of **unidentified gastrointestinal neoplasia.**

In addition, blood loss may be caused by **chronic intake of alcohol, salicylates, steroids and non-steroidal anti-inflammatory drugs.** In children, the most frequent cause of intestinal bleeding is **uncinariasis or trichocephaliasis** and chronic bleeding from hemorrhagic hereditary diseases such as **hemophilia** (**Chart 2**) [13].

CHART 2. ETIOLOGY OF IRON DEFICIENCY ANEMIA

1.- INADEQUATE IRON INTAKE

A) Inadequate diet
B) Accelerated growth
- **a) Premature birth**
- **b) Breastfeeding**
- **c) Adolescence**

C) Pregnancy

2.- ABSORPTION DEFECTS

A) Gastrointestinal *resection*
B) Gastrointestinal abnormalities
C) Malabsorption syndromes
- **a) Celiac disease**
- **b) Steatorrhea**
- **c) Sprue**

3.- CHRONIC BLOOD LOSS

A) Chronic bleeding of the digestive tract

- **a) Gastric ulcer, hyatal hernia, esophagic varices, polyps, diverticula-, hemorrhoids.**
- **b) Intestinal parasites: uncinarias, tricocephalus**
- **c) Chronic genital hemorrhage**
- **d) Chronic lung hemorrhage**
- **e) Hemorrhagic hereditary disease**

7.4 Clinical features

Ferropenic anemia is an insidious illness and the severity of its manifestation depends on the degree and velocity of development. At early stages of the deficiency, signs and symptoms of the disease are often not recognized until anemia is fully developed. The most frequent symptoms are pallor, irritability, weakness, tiredness, fatigue, lightheadedness, palpitations, headache and disturbed sleep.

When the evolution of the disease has been extended, compensatory mechanisms of the circulatory and respiratory systems are developed; clinical manifestations diminish and allow a higher

tolerance to low concentrations of hemoglobin. As the anemia get worse, it significantly limits work capacity and physical activity; it can lead to cardiorespiratory distress and even death. Some symptoms are considered characteristic of iron deficiency, such as: **glossitis**, **stomatitis, lingual papillae atrophy, gastric atrophy, oral mucosa inflammation with burning sensation of the tongue.** Changes on the tongue or oral mucosa are the cause of pica and **pagophagia** (compulsive ice eating). **Koilonychia** (thin, breakable and spooning of fingernails) results from the alteration of the growth of the epithelium of **nail bed** and it is a characteristic of iron deficiency.

It has been shown that iron deficiency, even without anemia, impairs growth and intellectual development in children at all ages. In infants and preschoolers it leads to neurocognitive deficit that may be irreversible when deficiency is prolonged. **Iron is necessary for oligodendrocytes and dopaminergic metabolism,** thus iron deficiency may cause **hypomyelination,** which could in turn explain the neurocognitive deficit.

Tamura et al [15] studied the association between fetal iron status (Ferritin concentrations in umbilical cord) and mental and psychomotor development at 5 years of age. They concluded that **decreased Ferritin concentrations *in utero* are associated with poor performance in mental and psychomotor tests.**

Lozoff and colleagues [16] demonstrated that children who had iron deficiency anemia during infancy are at an increased risk for a long-lasting, adverse neurodevelopmental outcome. Other authors have demonstrated that children who suffered iron deficiency, regardless of whether they had anemia, scored lower on standardized mathematical tests than did those who had normal iron status. **Neurologic complications of iron deficiency in preschool age and older children may be irreversible even with treatment** [12,16,17].

7.5 Diagnosis

Diagnosis of iron deficiency anemia is based on clinical history, physical examination, and laboratory findings. Symptoms and pallor may not appear until the hemoglobin level is less than 9 g/dl. Laboratory criteria for anemia diagnosis cover**: Decrease in hemoglobin levels, reticulocyte count, and the mean corpuscular volume of erythrocytes.**

Examination of peripheral blood smear shows **microcytosis and hipochromia.** In the first stage of iron deficiency without anemia, serum Ferritin is diminished, indicating depletion of iron store as **hipoFerritinemia.** In the second stage serum iron decreases resulting in decreased Transferrin saturation and the free erythrocyte protoporphyrin increases due to a reduction of iron delivery to the erythrocytes and heme production decreases as well. As a result, newly produced **erythrocytes are microcytic and hypochromic** [11] (**Chart 3**).

CHART 3. REFERENCE VALUES FOR THE DIAGNOSIS OF IRON DEFICIENCY ANEMIA IN INFANTS FROM 6 TO 12 MONTHS OF AGE

PARAMETER	REFERENCE VALUE
Hemoglobin	**<10.5 to 11 g/dl**
MCV	**<73 to 71 fl**
Ferritin	**< 10 to 12 µg/L**
Serum iron	**< 60 µg/dl**
Protoporfirin-Z	**>75 to 90 µmol/mol heme**
TfR	**>11 mg/L**

MCV, mean cell volume; TfR, Transferrin receptor.

7.6 Treatment

Treatment to the anemic patient consists of the investigation and correction of its origin. Iron deficiency is corrected using oral iron preparations as **ferrous sulfate, fumarate or gluconate** (**Chart 4**) [13]. The usual dosage ranges from 3 to 6 mg/kg/day of elemental iron administered until normal levels of hemoglobin are reached and continues for 2 to 3 months to replenish iron stores.

Some patients have difficulties tolerating iron salts because they may have gastrointestinal adverse effects such as ***gastritis and constipation***. Liquid iron causes long lasting staining of the teeth. **Polysaccharide iron** complex preparations are as effective as ferrous sulfate preparations and may cause fewer adverse gastrointestinal effects.

Parenteral iron (dextran iron) is only recommended in patients who cannot tolerate oral iron or cannot absorb oral iron because of gastrointestinal disorders. Dextran iron is utilized which may cause mild adverse reactions as fever, headache, pruritus, and nausea or life threatening reaction as anaphylaxis. A test dose should be used before infusion (**Chart 4**)

CHART 4. IRON SALTS USED FOR TREATMENT OF IRON DEFICIENCY ANEMIA.

IRON SALTS	ELEMENTAL IRON (%)	THERAPEUTIC DOSES (mg / Kg weight)
FERROUS SULFATE	20	30
FERROUS GLUCONATE	12	40
FERROUS FUMARATE	33	15

8. Iron Overload

Hereditary hemochromatosis is a group of disorders characterized by **excessive intestinal absorption of iron from the diet, leading to accumulation of iron in the body and eventually to the saturation of Transferrin, Ferritin, and Hemosiderin and the abnormal deposition of iron in vital organs.**

The specific genetic defects that cause these groups of disorders have been discovered over the last decade. The most common form of hereditary hemochromatosis is presented in the **Northwestern European population** who are descendants of a common Celtic ancestor who lived 60 to 70 generations ago. They carry a unique **missense mutation** [nucleotide 845 that leads to a substitution of a tyrosine (Y) by cysteine (C) in amino acid position 282 (C282Y)] **in the hereditary hemochromatosis gene, which has a recessive autosomal inheritance of low penetrance.** This condition affects mostly men between 40 to 70 years old.

Other causes of hemochromatosis with similar phenotype have been described: **mutations in TfR2** (Receptor 2 of Transferrin); **mutation in the hepcidine gene HAMP** (hepcidine antimicrobial peptide); **mutation in the Hemojuvelin gene** most often causes a much more severe phenotype, which affects young men and women equally. **Mutation in Ferroportin gene** is transmitted as autosomal dominant and causes early iron overload in **Kupffer cells** (**liver macrophages**) rather than in hepatocytes [1,7]. There are less frequent iron overload disorders such as **neonatal hemochromatosis** and **African siderosis** of unknown etiology.

8.1 Pathogenesis

It now appears that most forms of hemachromatosis are due to **Hepcidin deficiency**, and the autosomal dominant form is due to the **dysfunction** of the main target of hepcidin, the cellular iron exporter

Ferroportin. In acquired iron overload due to frequent transfusions, urinary hepcidin excretion and production are increased, while in hereditary hemochromatosis several studies have indicated that hepcidin is low. This would suggest that the HFE gene is required for normal regulation of hepcidin synthesis and that the deleterious effects of HFE mutations are caused by hepcidin deficiency [7].

8.2 Clinical features

Patients with hemochromatosis regularly absorb 2 to 3 fold dietary iron than normal people. Most do not have symptoms until adulthood, although the saturation of serum Transferrin is usually increased since adolescence. **Absorbed iron excess is deposited in parenchymal cells of liver, heart, pancreas, pituitary gland and parathyroid gland.** Early symptoms are non-specific; they include fatigue, arthralgias, erectile dysfunction, and increased skin pigmentation. As the disease progresses, tender hepatomegaly develops and leads to liver fibrosis, and finally cirrhosis.

There is an increased incidence of **hepatocellular carcinoma** after substantial damage to the liver has occurred. Iron deposition in the heart causes **cardiomyopathy** that is usually congestive but may be restrictive or associated with pericarditis and arrhythmias. Most frequent endocrinopathies are: diabetes mellitus, hypopituitarism, hypogonadism, and hypoparathyroidism. Patients with hemochromatosis are more susceptible than others to infections.

8.3 Diagnosis

Hemochromatosis should be suspected when the **serum Ferritin saturation exceeds** 50% in premenopausal women and 60% in men and postmenopausal women. Serum Ferritin rises until more advanced stages of the iron overload, to levels higher than 300 ng/ml in men and than 200 ng/ml in women. This nonspecific parameter must be interpreted with caution since elevated serum Ferritin may result from infections, inflammation such as hepatitis, active arthritis or neoplastic diseases as neuroblastoma or histiocytosis.

Magnetic resonance may provide a technique to quantify the three-dimensional distribution of excess of iron in the body.

Liver biopsy is considered the most useful method to assess liver iron deposits (In hepatocytes and macrophages) and to obtain an estimation of total body load. Concentrations less than 1.4 mg/g of dry weight liver tissue are considered normal, whereas values that are greater than 2.4 mg/g are indicative of iron overload.

Genetic diagnosis is achieved by mutational testing of the HFE specific gene. Presence of the C282Y mutation and biochemical signs of iron overload confirm the diagnosis of hereditary hemochromatosis [1].

8.4 Treatment

Treatment for hereditary hemochromatosis has not changed in the last 50 years, and the **therapeutic phlebotomy** remains the most effective therapy for removing excess iron. Treatment must be initiated before symptoms develop, when Ferritin levels are higher than 200 or 300 ng/ml, starting with weekly phlebotomies of 500 ml of whole blood until serum Ferritin is lesser than 20 ng/mL. Thereafter, it is continued as needed to keep the serum level below 50 ng/mL. On average, men require phlebotomy three to four times per year, and women require one to two times per year. In addition to performing phlebotomy, **dietary recommendations** must be advised. Patients should avoid iron supplements, limiting red meat because its high levels of heme iron, and restrict their intake of vitamin C, which facilitates iron absorption.

When phlebotomy is instituted before end-stage organ damage has occurred, patients can have a normal life expectancy. When treatment is started late, symptomatology may improve, but **endocrine abnormalities and liver fibrosis are irreversible.**

Transfusional Siderosis.- Long-term transfusion therapy is routine for patients with hereditary hemolytic anemias (**thalassemia, sickle cell disease**), bone marrow failure anemia **(aplastic anemia, Fanconi's anemia**) or aggressive treatment of cancer.

As a result of the lack of mechanisms for iron excretion, repeated transfusion leads to iron overloading because each unit of blood contains 200 to 250 mg of iron. Since this iron is derived from red cells, reticuloendotelial macrophages become iron-loaded before the tissue cells of heart and endocrin glands. Cardiomyapathy is more prominent in patients with transfusional iron overload than in those with hemochromatosis.

The body iron burden is best determined by quantitative liver biopsy. This iron overload must be treated by **chelation therapy** administrated intravenously or orally to maintain a hepatic burden of less than 15 mg/g of liver dry weight [3].

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Chapter 3

Iron and Oxidative Stress

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1. Abstract

Iron is a transition metal needed for physiological function of cells. However, alterations in the metabolism cause pathological conditions.

Iron plays an important role in generation of hydroxyl radical by Fenton reaction. This is one of the most important mechanisms of cell damage induced by iron.

Hydroxyl radical is a highly reactive radical and consequently short-lived, and cells do not have enzymatic defense to take it out. Due to its high reactivity, hydroxyl radical is able to react with lipids, carbohydrates, proteins and DNA causing cell homeostasis disruption, which could lead to cell death.

This chapter presents iron as an important mediator in the hydroxyl radical generation and the oxidative stress as a consequence involved in the pathophysiology of neurodegenerative, respiratory and renal diseases, as well as diabetes.

Key words: Fenton reaction, hydroxyl radical, iron, oxidative stress

2. Introduction

Iron is a transition metal taken by diet in a quantity of 3-4 g per day and absorbed in much less quantity by the small intestine. **The physiological functions of iron include metabolic regulation, cell growth and apoptosis.** However, **iron deficiency or iron excess is related to pathological processes** [1].

Intracellular iron homeostasis is mainly controlled by iron-regulatory proteins 1 and 2 (**IRP1 and IRP2**) which bind to RNA stem loops known as iron-responsive elements (**IREs**) and this binding is in charge of posttranscriptional regulation of iron metabolism genes, including **Transferrin receptor 1** (**TfR1**) and **Ferritin H and L subunits** [2; 3; 4]. For instance, **IRP represses Ferritin expression in iron-deficient cells avoiding iron storage by Ferritin and at the same time, IRP stabilizes TfR1 mRNA** and TfR1 mediates iron uptake and when cells are sated of iron, the opposite response is observed. The complex mechanism of regulation in iron uptake and release shows that alterations in this regulation could be the origin of a pathological condition.

3. Oxidative stress and reactive oxygen species

Oxidative stress is an unbalance between reactive oxygen species (**ROS**) **and the antioxidant defense** [5]. This unbalance may be attributed to an increase of ROS, a decrease in antioxidant defense or both. On the other hand, **ROS are highly reactive molecules and they can be formed by univalent reduction of oxygen in the cell** (**Box 1**). They are toxic for the cell because they can modify lipids, carbohydrates, proteins and DNA.

The superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($HO^{\bullet}$) are the main ROS.

BOX1. FORMATION OF REACTIVE OXYGEN SPECIES

$$O_2 \xrightarrow{e^-} O_2^{\bullet-} \xrightarrow[2H^+]{e^-} H_2O_2 \xrightarrow{e^-} HO^{\bullet} + HO^- \xrightarrow[2H^+]{e^-} 2H_2O$$

The $\mathbf{O_2^{\bullet-}}$ is formed spontaneously in aerobic environments, including mitochondria, in the electron transport chain, due to one electron addition to the molecular oxygen. **This specie is an anion and radical at the same time, and it is also produced by the NADPH oxidase, Xhantine oxidase, Nitric oxide synthase, Lipoxygenase and Cyclooxygenase** [5; 6]. The $O_2^{\bullet-}$ can react with lipids in the membrane and it causes it

permeability and this anion and radical is also precursor of H_2O_2, which is also formed by two-electron addition to oxygen, but it is also formed by **Superoxide dismutase (SOD)** activity.

H_2O_2 has physiological functions, for example, some enzymes, such as **Myeloperoxidase in the neutrophils uses H_2O_2 to form hypochlorous acid during respiratory burst** [7] and it is also a signaling mediator [8]. H_2O_2 is not a radical molecule but it is the $HO^{\bullet}$ precursor, which is the highly reactive specie compared with other ROS [5; 9]. $HO^{\bullet}$ oxidizes lipids, carbohydrates, proteins and DNA [10] and sustained $HO^{\bullet}$ production can cause cell death.

Despite the endogenous ROS production, **cell has antioxidant mechanism to remove these oxidant species.** The antioxidant defense can be **divided into non-enzymatic and enzymatic systems.** The non-enzymatic system is formed by compound that can have one or more characteristics, including their ability to reduce oxidized compounds and/or oxidized antioxidants, or to prevent lipid peroxidation. In this category is found **reduced glutathione,** which is synthesized from the amino acids L-cysteine, L-glutamic acid and glycine and it also can be obtained by diet [11]; the **ascorbic acid, also named vitamin C,** is a non-enzymatic compound which must be obtained by diet. The antioxidant properties of vitamin C include the scavenger $O_2^{\bullet-}$ properties avoiding the oxidation of lipids and proteins and **it is also able to regenerate the alpha-tocopherol, a fat-soluble vitamin that prevents lipid peroxidation** [12].

On the other hand, the enzymatic antioxidant system is formed by the **SOD,** a member of metalloenzymes which catalyzes the conversion of $O_2^{\bullet-}$ into H_2O_2; the **Catalase,** a hemoprotein that catalyzes the conversion of H_2O_2 into water; the **Glutathione peroxidase,** which catalyzes the decomposition of H_2O_2 or organic peroxides using reduced glutathione as cofactor and as a consequence, the oxidized glutathione is produced. The **Glutathione reductase regenerates GSH and establishes a redox cycle** [9].

The **NADPH,** produced by **Malic enzyme** and **Glucose-6-phosphate dehydrogenase,** is needed also for the GSH recycling. In turn, NADPH is needed for the activity of **Heme-oxygenase 1 (HO-1),** the rate-limiting enzyme in Heme degradation, which is also an antioxidant enzyme present in the endoplasmic reticulum. **HO-1 catabolizes Heme into carbon monoxide (CO), iron and biliverdin,** and **this product turns into bilirubin by Biliverdin reductase** using NADPH as cofactor [13; 14]. **Bilirubin is a potent antioxidant that protects cells from H_2O_2** [15]. All the above information shows that cells have a wide antioxidant system to protect against oxidant species; however, there is no enzymatic defense to protect from $HO^{\bullet}$. The **Box 2** shows some antioxidant enzymes and their reactions.

BOX 2. REACTIONS OF ANTIOXIDANT ENZYMES

ENZYME	REACTION
Superoxide dismutase	$O_2^{\bullet -} + O_2^{\bullet -} + 2H^+ \rightarrow H_2O_2 + O_2$
Superoxide reductase	$O_2^{\bullet -} + 2e^- + 2H^+ \rightarrow H_2O_2$
Catalase	$H_2O_2 + H_2O_2 \rightarrow 2\,H_2O + O_2$
Glutathione peroxidase	$H_2O_2 + 2GSH \rightarrow GSSG + 2H_2O$ $ROOH + 2GSH \rightarrow ROH + GSSG$
Glutathione reductase	$GSSG + NADPH + H^+ \rightarrow 2GSH + NADP^+$
Tioredoxin reductase	$Trx\text{-}S_2 + NADPH + H^+ \rightarrow Trx\text{-}(SH_2) + NADP^+$ $Trx\text{-}(SH^2) + prot\text{-}S_2 \rightarrow Trx\text{-}S_2 + prot\text{-}(SH_2)$
Peroxiredoxins	$H_2O_2 + Prx\ (red) \rightarrow H_2O + Prx\ (ox)$
Hemo oxygenase	$Heme + O_2 \xrightarrow[NADPH + H+ \quad NADP+]{} CO + Biliverdin \xrightarrow[NADPH + H+ \quad NADP+]{} Bilirrubin$

The univalent reduction of oxygen to water involves the formation of HO•, however, the HO• is also formed by Fenton reaction. In 1894, Henry John Horstman Fenton described that some metals, including cooper and iron, were able to catalyze the HO• formation (**Box 3**). In this regard, iron plays a central role in the generation of HO•, which in turns could lead to oxidative stress in the cell and contribute to pathological environments.

BOX 3. INVOLVED REACTIONS IN HYDROXYL RADICAL GENERATION

Haber-Weiss reaction	$Fe^{2+} + O_2 \rightarrow Fe^{3+} + O_2^{\bullet -}$ $O_2^{\bullet -} + H_2O_2 \rightarrow O_2 + HO^- + HO^{\bullet}$
Fenton Reaction	$Fe^{2+} + 2H_2O_2 \rightarrow Fe^{3+} + HO^- + HO^{\bullet}$

4. Alzheimer disease

Alzheimer's disease is characterized by the accumulation of β-amyloid peptides as plaques in the brain. These peptides are produced from amyloid precursor protein by sequential cleavage of **β- and γ-Secretase.** At the same time, it has been reported that **iron is accumulated in the brain regions affected by Alzheimer disease.** The free iron is involved in HO• formation, which causes lipid peroxidation, and this cellular event is

observed in Alzheimer patients. **Malondialdehyde is one of the end products of lipid peroxidation, and this aldehyde has been found in the serum of Alzheimer patients** [16].

In addition, **4-hydroxy-2-nonenal, another lipid peroxidation end-product is found in brain of experimental model of Alzheimer disease** [17]. Several experiments have demonstrated that lipid peroxidation is involved in Alzheimer disease and $HO^{\bullet}$ generation is a central mediator of this process. Indeed, **iron chelation has been proposed as a therapy for Alzheimer disease since it has demonstrated that iron is responsible, at least in part, for the stabilization of β-amyloid plaques** [18]. Surprisingly, after β-amyloid plaques formation, less iron neurotoxicity is observed [19] and this effect could be attributed to the reaction between β-amyloid plaques and iron avoiding iron-catalyzed $HO^{\bullet}$ generation [20]. ROS and β-amyloid plaques promote activation of microglia, which is formed by immunocompetent cells that have similarities with macrophages [21]. **The microglia activation involves iron storage, which in turn, contributes to microglia degeneration by increase in oxidative stress** [22].

5. Friedreich ataxia

Friedreich ataxia is an autosomal recessive progressive neurodegenerative disorder caused by a large GAA triplet repeat expansion within the first intron of the gene encoding Frataxin [23]. The consequence of this mutation is reduced or lacking levels of mature Frataxin, which is an iron storage protein [23].

Frataxin mutation causes insufficiency of iron-sulfur cluster-containing subunits of the mitochondrial electron transport complexes I, II, and III, and of the iron-sulfur protein **Aconitase** [24] and as a consequence, decrease in ATP synthesis. In addition, **Friedreich ataxia disease also causes an excess of iron in mitochondria** and an increase in $O_2^{\bullet-}$, due, at least in part, to the decreased SOD activity [25]. The increase in H_2O_2 is also observed [26; 27] and in presence of iron, $HO^{\bullet}$ is generated via the Fenton reaction [28]. In addition, **in Frataxin absence, iron is stored by Transferrin in mitochondria increasing the iron traffic from cytosol to mitochondria** [29].

As we can see, **one of the most important alterations in Friedreich ataxia is the mitochondrial unbalance and the following alteration of the electron transport chain leading to oxygen decreased consume in the cell and the high susceptibility to oxidative stress** [30; 31]. In this regard, this high susceptibility is an adjunct to the decrease in glutathione content [25 32]. In addition, Friedreich ataxia disease has been related to tumor development and the answer is found in the fact that DNA repair enzymes contain iron-sulphur-clusters [33].

On the other hand, **Frataxin is a protein expressed in high oxygen consumption tissue, including heart and brain.** This is the reason why cardiac and nervous systems are affected in Friedreich ataxia. Since

Frataxin deficiency could lead to an increase in iron release and $HO^{\bullet}$ generation by Fenton reaction, **the iron scavengers have been used as potential drugs against Friedreich ataxia.** However, the toxicity and inefficiency to cross cell membrane are some of problems with new drugs. Hence, **2-pyridylcarboxaldehyde 2-thiophenecarboxyl hydrazone (PCYH) has been studied as an iron chelation therapy.** This compound has shown to prevent the decrease in cell viability of fibroblast induced by H_2O_2, more than other iron chelators such as deferiprone and desferrioxamine [34].

6. Diabetes

Diabetes is a chronic disease caused by an insulin deficiency named type-I Diabetes, but there is also possible that **the amount of insulin could be inefficient to generate a physiological glucose metabolism, which is called type-2 Diabetes.** In this sense, **insulin resistance is a common feature of Type-2 diabetes,** which is related to a less efficiency of insulin to decrease blood glucose concentration.

Interestingly, there is evidence that **the prevalence of iron overload is higher in patients with insulin resistance** and an association between iron overload and obesity, besides it was higher Ferritin levels in subjects with insulin resistance than control patients [35]. It could be possible that higher Ferritin levels are related to higher iron overload in these patients. However, the increase in serum Ferritin concentration is observed even in pre-diabetic patients and those patients had positive correlation between serum Ferritin and plasma glucose, triglyceride, cholesterol and blood pressure [36].

Besides these positive correlations, **there is also a negative correlation between serum Ferritin and HDL2 cholesterol** [37]. Taken together, the increase in serum Ferritin enhances the risk to overload iron and the possibility that several tissues were exposed to iron. As an example, liver is affected by high iron concentration in patients with insulin resistance because it is deposited in this tissue [38]. **Some of the iron deposition effects in the liver include the decrease in glucose uptake by Glut1 transporter and the decrease in mRNA of insulin receptor.** Opposing, the iron depletion in rats showed an improved glucose uptake by increasing insulin receptor and signaling in hepatocytes [38].

On the other hand, **erythrocytes from type-2 diabetic patients are related to an increase in oxidative stress makers including lipid peroxidation** [39], **F-2-isoprostane** [40] **and also in the levels of antioxidant enzymes** [39]. **The maturation of reticulocytes to erythrocytes is also affected** [39]. It has demonstrated iron can be released from Hemoglobin [41] as a consequence of Hemoglobin oxidation [42] and the increase in glucose concentration [43] and it could participate in $HO^{\bullet}$ production by Fenton reaction. Since $HO^{\bullet}$ is strongly related to lipid peroxidation, it could be possible that iron will be involved in oxidative stress generation in erythrocytes from diabetic patients.

The increase in lipid peroxidation is also clear in hemodialysis patients, since administration of intravenous iron preparations during hemodialysis **increases malondialdehyde** more pronounced in diabetic patients [44]. The above information suggests that iron could be an important mediator in the red blood cell damage in diabetic patients.

On the other hand, data from the World Health Organization show that more than 400 million of adults are clinically obese and **obesity is closely linked to type-2 diabetes.** Recently, it has been found that one of the metabolic alterations present in obese individuals is the increase in urinary iron compared to non-obese individuals [45]. **The role of iron in diabetes seems to be more important than it had been proposed** and more studies are needed to investigate the long-term consequences of iron release in diabetes.

7. Respiratory diseases

There is increasing evidence about adverse health effects of **airborne particulate matter (PM)** and one of the most important are related to respiratory diseases including lung cancer [46; 47; 48]. However, the list of diseases related to particulate matter is growing and there is evidence about potential side effects on cardiovascular system [49]. PM of an aerodynamic diameter <10 mm (PM_{10}) is derived from anthropogenic activity and deposits in the thoracic region, while some PM of an aerodynamic diameter <2.5 mm ($PM_{2.5}$) deposits in alveolar region of the lung.

PM is a heterogeneous mixture of organic and inorganic pollutants that includes dust, soot, liquid and aerosol particles, organic carbon, polycyclic aromatic hydrocarbons, endotoxin and metals such as iron, lead, potassium, titanium, vanadium and copper [50]. It is remarkable that **iron is one of the main components of PM, and it has demonstrated that it is responsible for oxidative effects.** Specifically, the PM_{10} exposure in lung epithelial cell causes DNA damage, the activation of DNA repair machinery and in the activity of **p53** and **these effects are partially prevented by trolox incubation** [51]. The PM_{10} also induces a decrease in glutathione content and an increase in oxidized proteins and lipid peroxidation. These effects are also partially prevented by trolox addition [52].

The trolox protective effect is related to its iron chelation capacity and it demonstrated that iron is responsible, at least in part, of the adverse cellular effects of PM_{10}. Those experiments showed no changes in cell viability after PM_{10} exposure; however, cell culture cannot survive after a second oxidative challenge [52]. The above information shows that PM_{10} can induce oxidative stress without affect cell viability and but also predispose cells to further cell damage induced by oxidants with iron as a central metal involved in the mechanism of cell damage.

Cigarette smoking is considerated as a risk factor for lung cancer and iron also has changes in its metabolism after its exposure, as it has been demonstrated an increase in iron and Ferritin concentration

of bronchoalveolar lavage in healthy smokers and also in smoking patients with **Chronic Obstructive Pulmonary Disease** [53]. The cigarette smoke is also able to induce an increase in divalent metal transporter-1 (**DMT-1**) expression in lung tissue of rats and this transporter increases with iron availability [53].

8. Acute kidney injury

The acute kidney injury (AKI) is a rapid breakdown of renal function that occurs as a consequence of decreased glomerular filtration rate and for this reason that some waste metabolic products are accumulated in blood stream, including creatinine and urea. **The causes of AKI include antibiotics** such as **gentamicin,** used against Gram negative infections, **and also antineoplasic agents** such as **cisplatin** [54], used against several types of cancers including head and neck, ovarium, testicle and lung [55].

The mechanism of renal damage induced by gentamicin and cisplatin treatments is related to the $HO^{\bullet}$ generation and some iron chelators are able to prevent cell damage [56; 57; 58]. For example, **desferrioxamine,** an iron chelator, inhibits free radical generation in renal cortex induced by gentamicin [59]. **S-allylmercaptocysteine,** a garlic-derived compound, has been also used as antioxidant against gentamicin-induced AKI since it is able to scavenge free radicals including $HO^{\bullet}$ [56]. Other experiments have demonstrated that iron release, observed in renal cell culture and also in renal tissue of cisplatin-treated animals, is associated with nephrotoxicity and with an increase in oxidative stress [60]. In order to avoid side effects of cisplatin and oxidative stress, a large amount of antioxidants and iron scavengers have been tested in experimental models. **Desferrioxamine** is able to prevent the increase in lipid peroxidation and H_2O_2 in kidney of cisplatin-treated rats [61].

9. Conclusion

The above information strongly shows that iron plays a central role in different diseases and one of the mechanisms of iron cell damage is related to the $HO^{\bullet}$ generation by Fenton reaction, which in turn, generates a pro-oxidant environment. Iron scavenger is proposed as a potential tool to prevent the oxidative stress induced adverse effects.

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Chapter 4

Iron storage: a matter of life or death!

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Abstract

Iron is an essential nutrient for all eukaryotes and for most of prokaryotes. However, its bioavailability is limited and it is highly toxic. Hence organisms have developed mechanisms to store it by keeping an iron intracellular deposit. Thus, it is rendered harmless to the cell and available at all times.

When iron concentration is too high, this storage performs an additional protective “antioxidant” function, by keeping the iron away from oxidants.

Organisms have evolved distributing the functions of iron storage and antioxidant in different ways among proteins like Ferritin, organelles like vacuoles, or even using more simple molecules like siderophores.

Regulation of the expression of these proteins, transporters or molecules, shows how important is the coordination between iron metabolism and protection against oxidative stress.

Key words: bacterial ferritin, bacterioferritin Dps, ferritin, frataxin, intracellular siderophores,.

1. Introduction

Iron is an **essential nutrient** for all eukaryotes and most prokaryotes. As a **transition metal**, iron has **redox properties** allowing it to exist in two oxidation states, **ferrous** (**Fe^{2+}**) and **ferric** (**Fe^{3+}**) for the donation and acceptance of electrons, respectively. This chemical property grants iron its role as an **essential cofactor** and **catalyst** in fundamental metabolic pathways [1], such as oxygen transport, electron transference, nitrogen fixation, DNA synthesis, and hemoprotein production like hemoglobin and myoglobin [2].

Therefore, **in order to survive, a sufficient iron supply is of paramount importance**. The value of iron to life is reflected in how abundant it is; since this metal is **the 4th most abundant element on Earth**. Now, if theoretically iron is not in limiting amounts, **why is it necessary to squirrel it away? Two reasons: its limited bioavailability and its potential toxicity.**

The **Fe^{2+} form is soluble in aqueous solution;** however, when oxygen is present **Fe^{2+}** is oxidized to **Fe^{3+}, which is extremely insoluble at physiological pH.** Therefore, Fe bioavailability is poor and frequently it is a limiting nutrient for growth [3]. As a result from its variation between ferric and ferrous state, iron is toxic. **Oxidization from Fe^{2+} to Fe^{3+} by oxygen generates reduced oxygen species like superoxide, hydrogen peroxide, and water.**

Hydrogen peroxide, an unavoidable byproduct of aerobic respiration, reacts easily with Fe^{2+} to generate **hydroxyl radical**, **$OH^{\cdot}$** (known as Fenton's reaction). This radical is **one of the most reactive species found in biological systems and attacks every kind of biological macromolecule, causing its abnormal function or inactivation.** Fe^{3+} can be reduced back to Fe^{2+} by cytoplasmic reducers and in some cases by superoxide, resulting in Haber-Weiss catalytic cycle [3].

Due to iron's poor availability and toxicity, organisms have evolved mechanisms to acquire this element and to keep it in a non-toxic form. Iron storage in a solubilized mineral form, within a protein coat, is a solution to both problems.

For decades, the role of iron storage and detoxification has been the synonym of **Ferritin** protein family, and it is not until recently that other proteins have been found to be capable of iron storage/detoxification, such as **DNA-binding proteins from starved cells (Dps)** and **mitochondrial Frataxin** [3].

In this chapter we describe protein and non-protein mechanisms that organisms use for iron storage and detoxification. **Table 1** shows a list of all of them.

2. Ferritin

Ferritin's main function is to store iron and to provide cells with a sufficient amount of it. When iron concentration is too high, Ferritin also has a **protective function as an "antioxidant"** by sequestering iron within its cavity away from O_2, hydrogen peroxide, and superoxide. In anaerobic organisms or in the anaerobic reaction centers of cells, Ferritin also has a **dioxygen detoxification** role, by consuming dioxygen through the **Ferroxidase reaction.** Under extreme conditions, iron detoxification or protection against O_2 and its radical products are secondary protective functions of Ferritin [4].

Ferritin is a ubiquitous protein. It is found in tissues, serum and other animal subcellular compartments (**Table 1**). Also, it is expressed in most eubacteria, archaea, plants, and some fungi, with the remarkable exception of yeasts [5]. **In humans, 15 to 20% of body iron is found in a storage form, as Ferritin or Hemosiderin.** Ferritin is the intracellular protein responsible for normal iron storage, while Hemosiderin is a degradation product of Ferritin that is predominant during iron overload conditions [2].

Table 1. Mechanisms of iron storage and detoxification

MECHANISM	ORGANISM	SUB-CELLULAR LOCALIZA-TION	SUBUNITS	SUBUNITS NUMBER	Fe ATOMS	IRON MINERAL	FUNCTION
PROTEIN							
FERRITIN	Animals Plants Fungi-zigomycetes Bacteria	Cytosol Nucleus Mitochondria Lysosomes Extracellular plastids	L chain (19 kDa), H chain (21 kDa), M chain (amphibians only) 4 in plants	24 (450 kDa)	1000-4500	Ferrihydrite (animals) Ferric-oxyhydroxide-phosfate	Storage Oxidative stress resistance
BACTERIO-FERRITIN	Bacteria	Cytosol	Homopolymers and a few heteropolymers	24	600-2300	Ferric-oxyhydroxide-phosfate	Oxidative stress resistance?
Dps	Bacteria	DNA-associated or no associated	Homopolymers	12	500	Microcrystalline tetrahedral symmetry	Oxidative- stress resistance DNA protection
FRATAXIN	Bacteria Yeast Animals	Mitochondria	14 kDa monomers Homopolymers	Several oligomeric states 3-48	2400	Ferrihydrite	Fe delivery to other proteins Storage Oxidative- stress resistance
NON PROTEIN							
INTRA-CELLULAR SIDEROPHORE (hydroxamate)	Fungi	Cytosol	Ferricrocin Hydroxyferricrocin M_r <1500	-	1	Chelation	Storage Oxidative- stress resistance
VACUOLE	Plants Fungi	Cytosol	-	-	-	Ferric Polyphosphate	Storage Oxidative- stress resistance

3. Conservation of Ferritin structure

Ferrintins have a **highly conserved structure**, in which 24 subunits are assembled to form a hollow protein shell approximately spherical, with a molecular weight of 450 kDa [6]. There are two types of protein subunits, **H subunit (21 kDa) and L subunit (19 kDa)**, and they are encoded by different genes. Each subunit is folded in four parallel long α-helixes with a loop and a fifth short helix in the carboxyl terminal forming a cylindrical subunit. Secondary structure of both subunits is very similar despite the fact that they only share 55% of sequence identity [2]. In fact, **the structure of this family of molecules is highly conserved among eukaryotes and prokaryotes, although sequence identity is as low as 15%** [5].

4. Ferritin in Bacteria

There are three members of the Ferritin family in bacteria: **Bacterial Ferritin,** similar to eukaryotic Ferritin, **Bacterioferritin,** which has a heme group and is found only in eubacteria, **and the small proteins Dps** only found in prokaryotes. Interestingly, **the tree types can be found in the same bacteria** [7].

4.1 Bacterial Ferritin

Bacterial Ferritins seem to have 24 identical subunits, similar to H subunit from eukaryotes, although the existence of heteropolymers is possible in some species [3]. The most studied is the Ferritin A of *Escherichia coli*. Studies inactivating this gene (***ftnA***) suggest that the **FtnA function is to accumulate iron during post-exponential growth** in the presence of iron excess and to use it as an intracellular source of iron during subsequent growth in iron deficiency conditions. Therefore, FtnA fulfills the classic role of iron storage from higher eukaryotic Ferritins, even though a role of FtnA in iron detoxification or in oxidative stress resistance has not been discovered [7].

***E. coli* also has a gene encoding a second Ferritin, FtnB, but it is not clear yet if it works like an iron storage protein, since it lacks an active ferroxidase center** [8].

In eukaryotic Ferritin, Fe^{2+} oxidization uses O_2 as a substrate; as a result of this ferroxidation reaction, H_2O_2 is produced, which is a poor substrate for oxidation, and so the protein has to get rid of it subsequently. However, **bacterial Ferritins do not need to deal with an oxidant species like H_2O_2** as a byproduct, since theoretically they can use H_2O_2 as well as O_2 for Fe^{+2} oxidation [9]. Besides, the ferroxidase center is tri- or tetra-nuclear, meaning that it contains three or four iron binding sites. This changes the oxidation stoichiometry, leading to oxygen reduction up to water instead H_2O_2, which is toxic [3].

It has also been found that Ferritins from other bacteria (*Campylobacter jejuni*, *Porphyromonas gingivalis)* have a role in increasing growth in conditions of iron deprivation; hence they seem to act mainly

as iron storage proteins. *C. jejuni* and *Helicobacter pylori* mutants in the Ferritin gene were more sensitive to oxidative stress or metals, suggesting that these Ferritins are able not only to detoxify but also to store iron [7,8].

4.2 Bacterioferritin

Although Bacterioferritins (**Bfr**) are more common in bacteria than Ferritins, the physiological role of these proteins is less clear [7]. **They are composed of 24 subunits "eukaryotic H-like" and in most cases homopolymeric, with the exception of** some organisms like *Pseudomonas aeruginosa*, which Bfr has two subunits. These proteins share the same general structural characteristics of eukaryotic and bacterial Ferritins [3]. **The difference lies in the fact that all Bacterioferritins have a heme group, in the form of protoporphyrin IX**, whereas Bfr from *Desulfovibrio desulfuricans* uses a new kind of heme coproporphyrin II - iron [7].

Usually, there are 12 heme groups in a 24-mer localized in each of the 12 interfaces between two subunits, exposed towards the inner cavity. The role of heme group is still unknown. *In vitro*, Bfr variants without the heme group bind to iron at indistinguishable rates compared to wild type Bfr, suggesting that heme group is not required for iron intake. However, Bfr variants without heme group accumulate 4 times more iron *in vivo* than wild type, indicating that **heme group could be involved in mediating iron release from Bfr by facilitating iron core reduction** [7].

Another difference of Bfr is the iron mineral within the core, which contains larger quantities of phosphate, producing an "iron-oxihydroxide-phosphate" mineral, which is more amorphous than the crystalline core of "ferrihydrite" from eukaryotic Ferritin. In Bfr, two ferroxidase centers act in a concerted way to reduce one molecule of O_2 driving to an oxygen reduction to water instead of H_2O_2, as it happens in FtnA. Additionally, H_2O_2 oxidizes Fe^{2+} in the ferroxidase center a lot faster than oxygen [3].

There are a few Bacterioferritins composed of heteropolymers. This is the case of *Neisseria gonorrhoeae´*s Bacterioferritin which subunit A gives it the ferroxidase activity and subunit B confers the ability to bind to the heme group [8].

During periods of iron sufficiency, up to 50% of cellular iron is sequestered by $FtnA_{EC}$, while less than 1% is found in $\mathbf{Bfr_{EC}}$ indicating that at least in *E. coli* Bfr is not involved in storage and does not function as an iron source to the cell [8].

Until now, no phenotype has been identified for the *bfr* mutant of *E. coli*, and it seems that Bfr protein does not play a major role in iron storage in other bacteria, like *Brucella melitensis*. However, the *bfrA* mutant of *P. aeruginosa* is sensitive to peroxides suggesting **a role for Bfr in redox stress resistance**, even though Bfr may not be directly involved, since it was speculated that Bfr could be an iron source for the manufacture of heme groups incorporated into catalase [7]. Something similar happens with a Bfr B mutant of

N. gonorrhoeae, which is sensitive to peroxide and **paraquat** (generates superoxide in bacterial cytosol), but additionally this bacterium has a reduced growth compared to wild type, indicating that Bfr B could be an iron source during iron deprivation [10].

4.3 Bacterial Ferritin and Bacterioferritin regulation

Regulation of the expression of Ferritin´s family in bacteria is linked to its main functions: iron storage and detoxification, or protection against O_2 and its radical products. Therefore, **the basic stimuli regulating its expression are iron availability and oxidative stress response. In bacteria and plants, regulation occurs only at transcriptional level**, **while in mammals regulation is both transcriptional and post-transcriptional** [2].

FtnA expression profile matches the physiological role of FtnA, since it is iron- and exponential growth phase-induced [7]. **Bacteria typically regulate its iron metabolism in response to iron availability**; in *E. coli* and other bacteria this regulation is mediated by Fur (ferric uptake regulator). **Fur works as a global regulator of iron homeostasis controlling the induction of iron intake functions** (during iron deprivation) **and expression of iron storage proteins like Ferritin and iron using enzymes** (during iron sufficiency) [11].

Fur_{EC} (Fur of *E. coli***) has been implicated as a positive and negative regulator of gene expression.** Fur_{EC} is a homodimer, which **in iron presence functions as its co-repressor, by binding to a "Fur box" in DNA and preventing access to RNA polymerase, resulting in downstream gene repression**. During iron deprivation, Fur_{EC} is not able to bind DNA causing a derepression [7]. But **in both Ferritin and Bacterioferritin, positive Fur_{EC} regulation is indirect, mediated by a Fur-dependent repression of a regulatory antisense small RNA (sRNA) named RyhB. This sRNA acts at post-transcriptional level to repress the translation of Ferritins gene, and is repressed by Fur_{EC} in an iron-dependent way**.

This means that, in iron deprivation, the sRNA is transcribed and able to decrease the level of target Ferritin mRNA, inhibiting its expression; whereas in iron sufficiency, sRNA transcription is repressed by Fur_{EC}-Fe and therefore Ferritin synthesis is allowed (Figure 1) [11]. The only gene known to be induced by iron through a direct interaction with Fur is the Ferritin gen in *H. pylori (pfr)*. *pfr* expression is repressed by the iron free form of Fur through the direct interaction of apo-Fur with *pfr* promoter in a new Fur binding site. While complex Fur-Fe formation results in a *pfr* derepression [12].

In some systems, Fur proteins can sense signals other than iron, e.g. oxidants level, and it can even bind to DNA in absence of iron [11]. **In fact, *fur* gen is induced in response to oxidative stress, which reveals that a connection between oxidative stress management and iron homeostasis is also present in bacteria** [7].

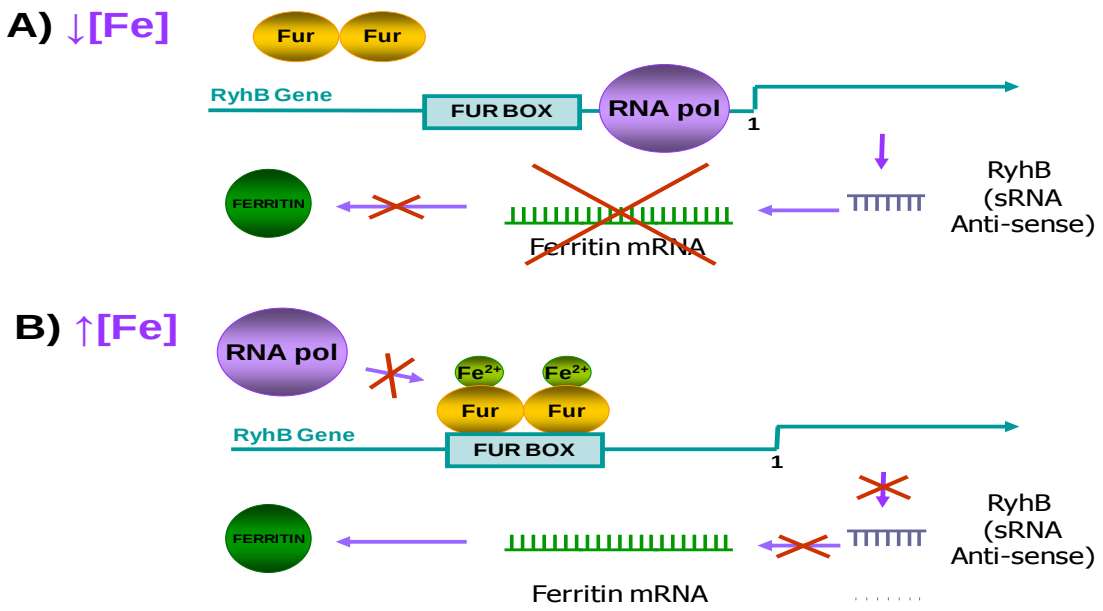

Figure 1. REGULATION OF BACTERIAL FERRITIN AND BACTERIOFERRITIN EXPRESSION BY FUR.

4.4 Dps

DNA binding proteins during starvation (Dps) are structurally very similar to Ferritin but smaller in size. **They are formed by 12 generally identical subunits**, producing a 250 kDa multimer, which is assembled to form **a hollow sphere with a cavity able to storage around 500 iron atoms per 12-mer**. Dps have three distinctive properties: (1) they bind nonspecifically to DNA, (2) the ferroxidase center is located in the interface between two subunits, instead of inside each subunit, as it happens in Maxiferritins, and (3) they use H_2O_2 as a substrate during iron oxidation, which prevents hydroxyl radicals production; thus eliminating the oxidative damage to bacteria, unlike the case of eukaryotic Ferritins where H_2O_2 is released [9].

Findings indicate that the most important role of Dps in *E. coli* is to protect DNA against the combined action of ferrous iron and H_2O_2 to produce hydroxyl free radicals. Hence Dps probably do not have a strict iron storage function in this bacterium [7].

No DNA binding function has been detected in Dps of *C. jejuni*, *Streptococcus mutans, or Bacillus spp*. Nevertheless evidence indicates that the iron binding capability of these proteins protects bacteria against H_2O_2-induced stress by preventing iron catalysis in H_2O_2 disruption, which produces reactive oxygen intermediates, like in *E. coli*. Even bacteria like *Streptococcus suis*, which allow the entrance of iron without needing for its growth, require a Dpr protein (**D**ps-like **p**eroxide **r**esistant) to protect themselves against the toxic effects of iron and H_2O_2 [8].

4.5. Dps regulation

Dps_{EC} transcription is induced during exponential growth by the protein OxyR. OxyR is activated by H_2O_2-caused oxidation and induces the transcription of genes required for defense against oxidative stress. Other Dps proteins are expressed in iron starvation conditions during late logarithmic phase (**$MrgA_{B.subtilis}$, $DpsA_{Synechococcus}$, nonheme $Ferritin_{Listeria\ innocua}$**) [13]. *B. subtilis* Mrg A is also induced in oxidative stress conditions, while its promoter is under Per R control. **PerR is a member of the Fur family which is sensitive to iron lost by H_2O_2,** which leads to an abolished DNA binding capacity and a subsequent Mrg A transcription [14]. In general, while Dps transcription seems to be induced by oxidative stress during exponential and/or stationary phase in iron limiting conditions, in those where iron is rich the transcription is blocked.

Dps regulation in the archaea *Halobacterium salinarum* is different from other Dps genes, since transcription decreases in iron limiting conditions and in oxidative stress, while it increases in iron rich growing conditions. *H. salinarum* Dps transcription profile seems to be the same for FtnA or Bfr in *E. coli* or for Pfr in *H. pylori*, behaving in that manner like a true Ferritin [13].

5. Iron storage in plants

Iron storage and buffering at sub-cellular level are crucial mechanisms that allow plants to deal with iron scarcity as well as iron toxicity. **Vacuoles and plastids have a key role in iron compartmentalization** [15].

Inside plastids, Ferritins can store an important fraction of cellular iron. Additionally, these proteins play several roles related to iron homeostasis during development or the response to environmental stress (**Figure 2**) [15]. Ferritins are much less characterized in plants than in mammals. *Arabidopsis thaliana´*s genome sequencing revealed the presence of **4 genes. Moreover,** 4 Ferritin genes have been identified in cowpeas; therefore, the presence of a Ferritin gene family might be something common in higher plants. Each Ferritin seems to have not only a ferroxidase center, but also a nucleation site similar to L chain. A newly synthesized plant Ferritin is significantly bigger than its mammalian counterpart, approximately 32 kDa. This is partly due to the **signal peptide that directs it to a plastid** [3].

Ferritin transcription in plants is induced by iron or nitric oxide (NO) during antioxidant responses, but also by changes in iron concentration associated to symbiosis during nitrogen fixation [16]. **Siderophores** are iron chelation complexes released specifically by microorganisms during iron deficiency. Their presence also induce Ferritin gene transcription, even in the absence of iron, apparently as a defense mechanism, using a regulation system different from the iron regulated one [16].

In pea seeds, it is widely proposed that Ferritin is the main form of iron storage, constituting up to 92% of stored iron, and that it provides iron for all iron containing proteins after germination. **Seed´s Ferritin**

is then essential to the proper construction of the photosynthetic apparatus and thus it is involved in autotrophic/heterotrophic transition. In leaves, it has been hypothesized that Ferritin is an iron source in early stages of development, used in the synthesis of iron-containing proteins involved in photosynthesis [17,18].

In *Arabidopsis*, however, estimated iron content in Ferritin represents no more than 5% of total iron in the seeds and it is not an iron source during development, and so the intracellular iron storage site may differ between legumes (such as pea) and *Arabidopsis*. **Ferritin may have only a minor role in iron storage in *Arabidopsis*, but it has a key importance in the defense machinery against oxidative stress induced by free iron** [18].

In *Arabidopsis*, vacuoles seem to be the most important iron storage site in seeds, according to studies involving mutants of vacuolar iron transporters. Here, it was observed that vacuoles are required for iron distribution in seeds and for iron re-mobilization during germination through **vacuolar exporters NRAMP3 and NRAMP4**, and **vacuolar importer VIT1** (**Figure 2**) [19,20]. In other organs, such as leaves and roots, the relative contribution of Ferritin in plastids and vacuoles in buffering cellular iron has not yet been determined [15].

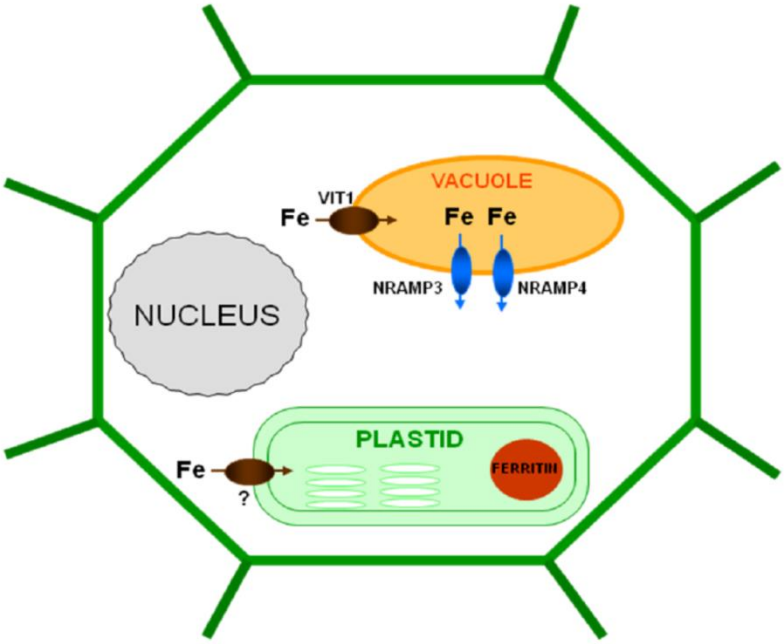

Figure 2. IRON STORAGE IN PLANTS

6. Iron storage in fungi

With the exception of zygomycetes, some of which produce **Mycoferritin**, other Ferritin-like molecules have not been described in fungi. Recently, a Ferritin-like protein was purified from *Aspergillus parasiticus.* However, the genome of this and other filamentous fungi do not appear to encode Ferritin-like molecules [21]. Two different mechanisms for iron storage in fungi have been described: **vacuolar and intracellular siderophore-mediated iron storage**.

6.1 Iron storage by intracellular siderophores

Siderophores, from the Greek "iron-bearing" are high-affinity chelating compounds, iron ion-specific and with a low molecular weight (<1500 Da). Their biosynthesis is controlled by iron bioavailability and their function is to provide iron to the cell. They may be excreted to internalize extracellular iron, or produced intracellularly mainly for iron storage. **Intracellular siderophores carry out the main functions of Ferritin: iron storage and iron detoxification**. Research on intracellular siderophores at the molecular level has been carried out with the intracellular siderophore **Ferricrocin,** in *Aspergillus nidulans*, *Cochliobolus heterotrophus*, *Gibberella zeae*, *Magnaporthe griseus* and *A. fumigatus* (**Figure 3**). A new intracellular siderophore, **Hydroxyferricrocin,** produced by *A. fumigatus*, is derived from Ferricrocin by hydroxylation [1].

Ferricrocin (or its hydroxylated derivate) account for 47%-74% of total iron in conidia of *A. nidulans*, *A. fumigatus*, *Aspergillus ochraceus*, and *N. crassa*, confirming its predominant role as an iron storage molecule [21]. Ferricrocin deficiency produces slow growth during iron starvation, delayed conidial germination and impaired sexual development [22]. A similar effect was observed in the sexual development of Ferricrocin-deficient *C. heterotrophus* [23]. **In *A. fumigatus*, Hydroxyferricrocin deficiency shows that intracellular siderophores are required for oxidative stress resistance and germination** [24].

6.2. Synthesis and regulation of intracellular siderophores

Ferricrocin and Hydroxyferricrocin are hydroxamates, like most siderophores, and derive from **ornithine**, a non-proteinogenic amino acid. They are classified inside the **ferrichromes,** consisting in 3 N^5-acil N^5-hydroxyornithine and three essential amino acids. The biosynthetic pathway starts with the **N^5-Oxygenase** (also known as N^5-hidroxilase), which catalyzes the N-hidroxylation of L-ornithine. The **N^5-Oxygenase** gene has been characterized in several fungi: *sid1* (*Ustilago maydis*), *sidA* (*A. nidulans*), *dffA* (*A.oryzae*), *sidA* (*A. fumigatus*), *sid1* (*F. graminearum*). Expression of these genes is repressed by iron. The next step is the hydroxamate group formation, with the addition of an acyl group from acyl coenzyme A by **N^5-Transacylase.** Finally the **Non-ribosomal Peptide Synthetases (NRPSs)** covalently link hydroxamate groups and sometimes other amino acids to complete siderophore biosynthesis.

NRPSs are large multifunctional enzymes that synthesize peptides by a thio-template mechanism, without the necessity of the ribosomal machinery nucleic acid-dependent. **NPRS** genes involved in Ferricrocin biosynthesis are *sidC* (*A. nidulans* y *A. fumigatus*), *NPS2* (*Cochliobolus heteostrophus* and *Fusarium graminearum/G.zeae*) and *SSM1* (*M. grisea*). Biosynthesis of Hydroxyferricrocin in *A. fumigatus* also required from *sidC* (since Ferricrocin is its precursor) [1].

In *A. nidulans*, Ferricrocin overregulation in iron excess conditions indicates that **Ferricrocin is the principal iron storage compound**. On the other hand, during iron starvation, high levels of Desferri-ferricrocin (without iron) were observed, probably as a proactive strategy to prevent iron-mediated oxidative stress from an expected rise in iron income. It has been shown that iron metabolism and oxidative stress are highly linked: (1) an increased Ferricrocin accumulation by oxidative stress and (2) Δ*sidC* conidia present an increased sensitivity to hydrogen peroxide. In *A. nidulans*, Ferricrocin seems to have a protection role against iron-induced toxicity [1,21]

SidC is regulated at transcriptional level by SreA repressor, which belongs to **GATA type transcriptional repressors**. These repressors bind to iron through a Fe-S group. This union is important for DNA binding to the GATA motifs localized within the gene promoter region. SreA represses genes involved in siderophores biosynthesis, like *SidC* under high iron conditions [1,21].

6.3. Vacuolar storage of iron

In contrast to most filamentous fungi and some yeasts like *Schizosaccharomyces pombe* [25], *S cerevisiae* and other yeast models like *Candida albicans and Cryptococcus neoformans* are not capable of synthesizing siderophores. However, they can use iron bound to siderophores produced by other microbial species (**xenosiderophores**). This is why *S cerevisiae* uses a different strategy for iron storage and iron detoxification [21].

S cerevisiae can grow for generations in absence of iron, which indicates its capability to store iron intracellularly. Several studies have suggested the importance of **yeast vacuole as the organelle for storage and detoxification of heavy metals, where iron is stored probably in its ferric form as a polyphosphate** [26]. **Ccc1p** mediates iron transport into the vacuole [27]. Iron export from the vacuole is supported by **Smf1p, paralog Smf3p**, and by a complex consisting of **Fet5p** and **Fth1p** that is paralogous to the Fet3p and Ftr1p complex, an iron multicopper oxidase (**Fet3p**) and a ferrous ion permease (**Ftr1p**) [1,21]. **Fre6p**, the vacuolar metalloreductase localized in membrane, provides iron to both vacuolar efflux systems (**Figure 3**).

Deficiencies in Smf3p or in the complex Fet5p and Fth1p cause signs of iron starvation, consistent with its role in mobilization of vacuolar iron storage. In contrast, Ccc1p lack causes sensitivity to iron. Fet5p and Ftn1p **orthologs** have been identified in *F. graminearum* and Ccc1p iron-regulated orthologs in *A. nidulans* and *S. pombe*, which suggests that this vacuolar iron storage is also present

in siderophores-producing fungi. Several fungi species, including *A. nidulans* and *S. pombe*, lack Fet5p and Ftn1p homologous, thus they must have a different way to mobilize vacuolar iron [21].

Vacuolar exporters Fet5p and Ftn1p, as well as Smf3p, are under Aft1 and Aft2 transcriptional control [28,29]. These activators bind to consensus sequences in the promoter region of the target gene in absence of iron to activate transcription. **Atf1 senses iron indirectly, probably through a mitochondrial product from Fe-S group biosynthesis, and in absence of iron it is translocated from cytosol to nucleus** [1]. On the other hand, messenger stability of the vacuolar importer, Ccc1p, is regulated by Cth1 and Cth2. Under iron–deficiency conditions, Atf1p induces Cth2 expression, which then binds to elements in the 3' untranslated region of the mRNA and destabilizes Ccc1p messenger. This results in a diminished expression of the transporter, thus preserving cytosolic iron levels [30]. Additionally, Ccc1p transcription is regulated by Yap5, a transcription factor which induces Ccc1p expression in high concentrations of iron [31].

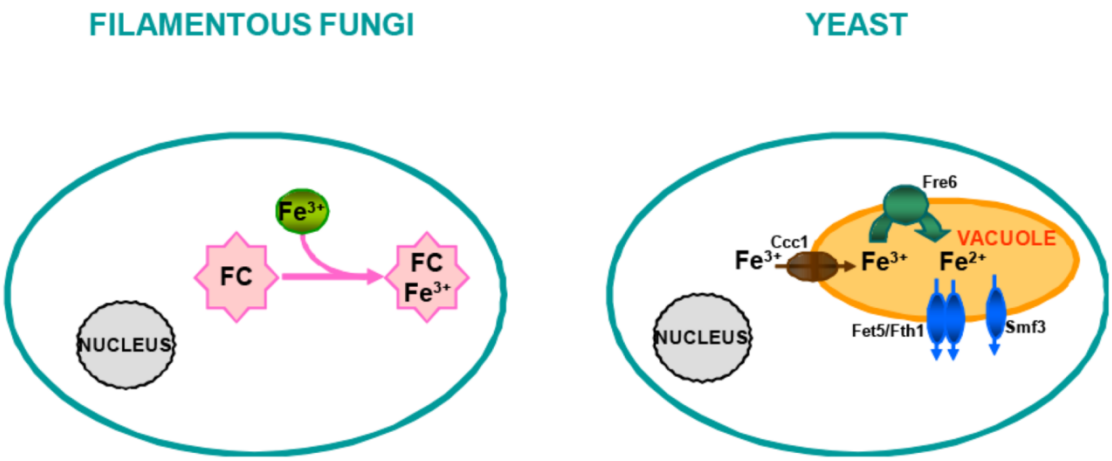

Figure 3. IRON STORAGE IN FUNGI

7. Frataxin

The importance of Frataxin in iron homeostasis was first demonstrated by observation of **Friereich's ataxia (FRDA), an autosomal inherited disease, linked to Frataxin mutations and dysfunction**. FRDA is a progressive disease in children and teenagers, and it is characterized by neurological damage, heart disease and diabetes *mellitus*. Most of FRDA patients have an extension in GAA repeats within the first intron of Frataxin gene, leading to abnormal structures in DNA that prevent gene transcription, causing Frataxin deficiency and iron homeostasis aberrations [25 32].

Frataxin is a mitochondrial, iron-binding protein, encoded in nucleus, conserved and ubiquitous from bacteria to humans, but without any homology to proteins with known functional domains. In humans, it is produced as a 30 kDa pro-peptide and a mature form of 15-17 kDa [33].

Frataxin family does not share an evolutionary-structural relationship with Ferritin family. Frataxin is folded into a α-β sandwich structure in which two helixes are packed against a β-sheet of five chains [34]. However, **there are similarities between Ferritin and Frataxin, like the general symmetry in the particles, the presence of pores at 3- and 4-fold axis of symmetry**. One of the characteristics that differentiate Frataxin from Ferritin is that **it has the unique ability to deliver iron to other proteins within mitochondria** [35].

Inside the Frataxin family there is a strong structural similarity, due to the fact that these proteins share an extremely high degree of similarity in the amino acids sequence. Sequence identity of **Yfh1** (yeasts), *versus* both **CyaY** (bacteria) and **HsFtx** (humans) are 59.8% and 65%, respectively [33]. Yfh1 Frataxin also possesses ferroxidase centers that use O_2 as an oxidant and produce H_2O_2, which apparently reacts with the protein, since it has not been detected during the reaction. CyaY uses H_2O_2 as oxidant instead [33]. It has been proposed that the ferroxidase center is formed by three subunits [3].

Unlike what happens with the Ferritin super family, Frataxin and its bacterial homolog CyaY can exist in several oligomeric states in solution, with variable quantities of stored iron. In the case of yeast Frataxin, each monomer is 14 kDa (**Yfh1**) and shows an iron-dependent assembly, which has been suggested to proceed through several stable intermediates: $\alpha \rightarrow \alpha_3 \rightarrow \alpha_6 \rightarrow \alpha_{12} \rightarrow \alpha_{24} \rightarrow \alpha_{48}$. The biggest oligomer is 840 kDa and may be composed of up to 2400 iron atoms. It has been suggested that stabilization and stepwise assembly of yeast Frataxin oligomers is mediated by the interactions among ferrhydrite crystals, formed in different mineralization sites. This suggestion is supported by the observation that yeast Frataxin oligomers can be easily disassembled into monomers after the reduction of its ferric iron core [35].

Unlike yeast Frataxin, human Frataxin (HsFtx) is assembled in an iron-independent way, presumably by interactions among subunits, mediated by a non conserved region of the protein at the amino terminus [35]. Frataxin ability of homo-oligomerization, forming aggregates with the capacity of binding iron, like Ferritin, suggests that Frataxin could act as an iron storage protein [33]. However, differences in oligomerizaton of Frataxins from different sources, tell us that they could have evolved to have different functions, probably all involved with Fe^{2+} delivery to processes that require iron, while only a few have the role in detoxification of oxygen reactive species and possibly iron storage.

The exact role of Frataxin in iron homeostasis and availability is still controversial. **It has been proposed that Frataxin participates in at least 5 different functions**:

1) As an **iron chaperone** during production of heme and Fe-S groups;

2) As an **iron storage protein** during iron overload;

3) As **auxiliar in repairing the oxidative damage** in Fe-S groups of aconitase;

4) As **a controlling stress factor** by moderating concentration of oxygen reactive species (ROS), and

5) As **an active participant in processes involving energy conversion** and oxidative phosphorylation.

A disruption in Frataxin production causes a general loss of cellular control of iron availability and its reactivity; however, phenotypes are complicated, thus preventing the description of the exact function of this protein [33].

Turning off the yeast Frataxin promoter in an inducible system caused accumulation of iron in mitochondria and resulted in mitochondrial and nuclear oxidative damage of DNA. The Frataxin-deficient yeast also showed increased production of hydrogen peroxide, higher sensitivity to DNA-damaging agents, and additional chromosomal instability, suggesting that **detoxification of mitochondrial iron is a main function of Frataxin.** Similarly, cells derived from FRDA patients had a 40% increase in mitochondrial iron content and were more susceptible to iron-mediated cytotoxicity and hydrogen peroxide, a phenotype that was rescued by the iron chelator **deferoxamine**.

These results support Frataxin´s function as an antioxidant through its capacity to either store iron, transport it or both. Surprisingly, expression of human mitochondrial Ferritin or human L-chain Ferritin rescued the phenotype of Frataxin-deficient yeast, suggesting a functional homology between the iron-storage protein Ferritin and Frataxin [36].

7.1 Regulation of Frataxin expression

Very little is known about Frataxin expression. **Frataxin presents a tissue–specific expression**, with more abundant mRNA in heart, liver and skeletal muscle. **3-nitropropionic acid and erythropoietin stimulate Frataxin expression in lymphocytes** derived from FRDA patients or normal people, even when the mechanism of this phenomenon has not been fully characterized. In mouse, the hypoxia-inducible factor (HIF-2α) specifically activates a hypoxia-responsive element (**HRE**) localized 2 kb upstream from the transcription-initiation site of the mouse Frataxin gene [36].

8. Conclusions

The main function of proteins and organelles that store iron is to maintain an intracellular iron deposit so that it is rendered harmless to the cell and is always available. When iron concentration is very high, they also have a protective “antioxidant” function by sequestering iron away from oxidizing agents like oxygen, hydrogen peroxide and superoxide, which can participate with iron in the generation of more radicals by the Fenton reaction. Ferritins, Bacterioferritins and Dps from different organisms have evolved distributing those functions, in many cases differently for each organism. The regulation of the expression of these

proteins demonstrates the importance of the coordination between iron metabolism and protection against oxidative stress.

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Chapter 5

Hemoglobin: ferrous protein of the blood

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1. Abstract

Hemoglobin (Hb) is the iron-containing oxygen transporter metalloprotein in the red blood cells (RBC) or erythrocytes of mammals and other animals. It carries oxygen from the lungs to all the body tissues and carries carbon dioxide (CO_2) and protons from the tissues to the lungs. In general, Hb can be saturated with oxygen (Oxyhemoglobin), or non-saturated (Deoxyhemoglobin).

Oxyhemoglobin is formed during physiological respiration when oxygen binds to heme component of Hb in the RBC. The heme group consists of an iron (Fe) ion (charged atom) held in a heterocyclic ring, known as porphyrin that is the prosthetic group. In general, the heme is important to the function of all aerobic cells and is involved in a variety of biological events by modulating the function or the state of hemoproteins.

Hemoglobin is the most abundant protein in RBC. When RBC reach the end of their life due to aging or some defects, the macrophages engulf and destroy these cells; the Hb molecule is disrupted and the iron gets recycled.

When hemoglobin is broken in blood, hemoglobin can dissociate in heme group and globin, in these conditions some serum proteins such as Haptoglobin is able to bind hemoglobin, and Hemopexin binds the heme group, then these complex proteins are transported to the liver. In this organ the heme is broken up by Heme oxigenase and the iron is released and is stored in Ferritin.

Key words: Heme, Heme-oxigenase, Hemoglobin.

2. Introduction

2.1. Heme group

Heme group consists of an iron ion sequestered in a heterocyclic ring, known as **porphyrin** that is the **prosthetic group**. The porphyrin ring consists of four pyrrole molecules cyclically linked together with the iron in its ferrous form (**Fe^{+2}**) bound in the center. **Heme is a prosthetic group of different types of proteins such as Hemoglobin, Myoglobin, Cytochrome c, Cytochrome p450, Catalase, and Peroxidase** [1]. In general, the Heme is important to the function of all aerobic cells and is involved in a variety of biological events by modulating the function and the state of hemoproteins.

For example, **protein synthesis is inhibited in erythroid cells under deficiency of Heme** as a consequence of the activation of the **Heme Regulated Inhibitor (HRI).** In contrast, the Fe concentration in the cell is regulated by the oxidation of Heme group and the consequent degradation of the **Iron Regulated Protein 2 (IRP2)** [1]. Importantly, the heme group determines its own fate; namely, **Heme regulates its synthesis and degradation through feedback mechanisms**, by which intracellular heme level is precisely maintained. Heme by itself reduces its synthesis by suppressing the expression of **non-specific 5-Aminolevulinate Synthase (ALAS 1)** and stimulates Heme breakdown by inducing **Heme Oxigenase (HO)-1** expression [1] (**Figure1**).

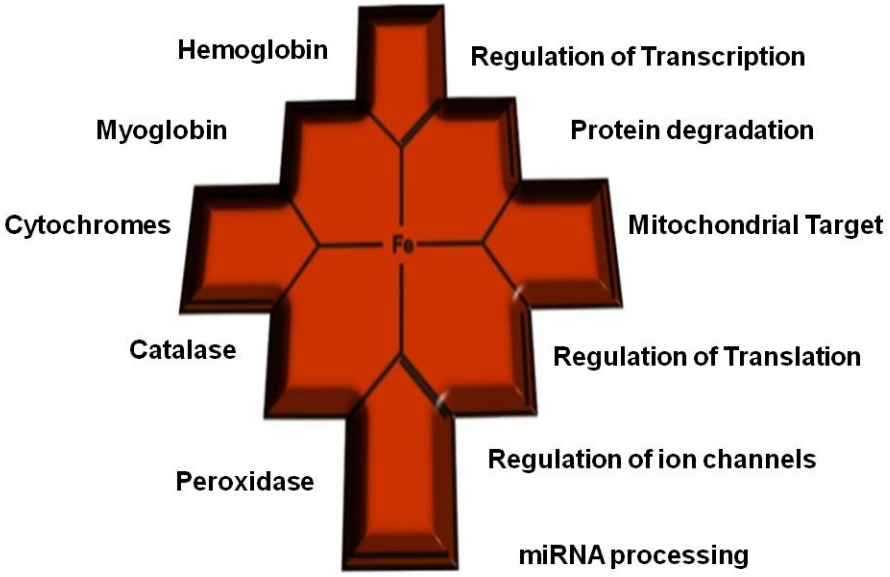

Figure 1. HEME PROSTHETIC GROUP. HEMOPROTEINS ON THE LEFT PART OF THEFIGURE; HEME FUNCTIONS ON THE RIGHT PART.

Taken from: http://www.ferato.com/wiki/index.php/Hemoglobina

2.2 Hemoglobin

Hemoglobin (Hb) is the iron-containing oxygen transporter metalloprotein in the **Red Blood Cells (RBC) or erythrocytes** of mammals and other animals. It carries oxygen from the lungs to all the body tissues and carries carbon dioxide (CO_2) and protons from the tissues to the lungs [2]. In mammals, Hemoglobin constitutes up to **97% of the RBC protein content.**

Hemoglobin synthesis begins in **pro-erythroblasts** and slightly continues even in the **reticulocyte** state, because when these cells leave the bone marrow and travel through the blood, they continue producing small quantities of Hb during around one day. **Heme is synthesized in a complex series of steps involving enzymes in the mitochondrion and in cell cytosol.**

The first step in Heme synthesis takes place in the mitochondrion, with the condensation of **succinyl CoA** (formed in the **Krebs cycle**) and **glycine** by **ALA synthase** to form **5-aminolevuleic acid (ALA)** [3]. This molecule is transported to the cytosol where a series of reactions produces a ring structure called **coproporphyrinogen III**, and then this molecule returns to the mitochondrion where an addition reaction produces protoporphyrin IX. The enzyme **Ferrochelatase introduces the iron into the ring structure of protoporphyrin IX to produce the Heme** [3]. On the other hand, two distinct globin chains (each with its Heme molecule) synthesized in the ribosomes combine to form Hb [3] (**Figure 2**).

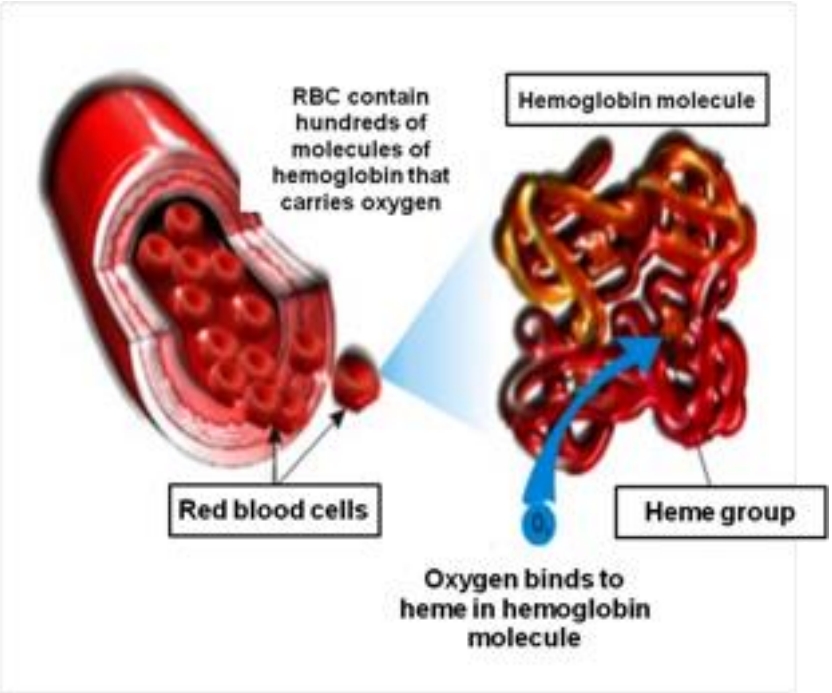

Figure 2. HEMOGLOBIN IS FOUND IN RED BLOOD CELLS WHICH TRANSPORT OXYGEN FROM THE LUNGS TO DIFFERENT TISSUES OF AN ORGANISM.

Taken from: http://www.ferato.com/wiki/index.php/Hemoglobina

The different types of Hb chains are called **alpha, beta, gamma, and delta. Hb A** is the most frequent form in adults; it is a tetramer formed by two pairs of polypeptidic chains that are known as **α and β chains**. Because each chain has one Heme prosthetic group, **there are four iron atoms in one single Hb molecule; therefore each Hb molecule is able to transport four oxygen atoms.**

In adult humans, the most common Hb type is a tetramer (which contains four subunit proteins) called Hb A, consisting of **two α- and two β-subunits non-covalently bound.** The α chain is composed by 141 amino acids with Mr of 15,126 daltons, whereas the β chain has 146 amino acids with Mr of 15,867 daltons. The subunits are structurally similar and have about the same size. Each subunit is able to bind one Heme group with Mr of 616 daltons; in this manner **the whole molecule has an Mr of 64,450 daltons.** This is denoted as $\boldsymbol{\alpha_2^A \beta_2^A}$**.** Hb A is the most intensively studied of Hb molecules [3] (**Figure 3**).

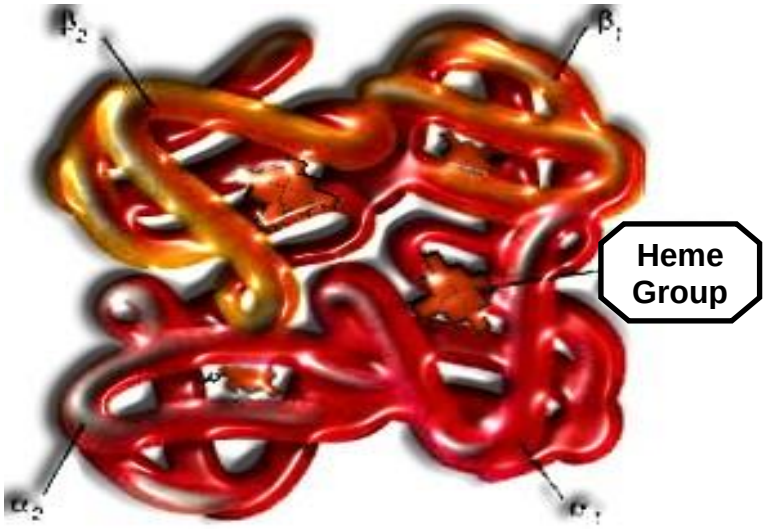

Figure 3. HEMOGLOBIN STRUCTURE A-1 AND A-2; B-1 AND B-2 CHAINS. EACH HEMOGLOBIN CHAIN BINDS A HEME GROUP.

Taken from http://www.ferato.com/wiki/index.php/Hemoglobina

The Heme residue of Hb, which is planar in three dimensions, is located in a cleft or pocket of each globin chain. It is anchored to the amino acid residues of globin largely through hydrophobic interactions involving its methyl and vinyl side-chains. **The iron present in Hb is of the ferrous type (+2), and is coordinated with the four nitrogen atoms of the protoporphyrin moiety, as well as with the imidazole residue.**

The imidazole residue is termed “proximal” and is located in position 92 of the beta and position 87 of the alpha chains. The sixth ligand of iron is oxygen in **Oxyhemoglobin,** which is also in contact with another imidazole residue of the globin termed “distal”. This is located in position 63 of the beta and 58 of the alpha chains. **Oxyhemoglobin may be oxidized to Metahemoglobin.** The ferric iron of Metahemoglobin is also coordinated with six ligands, but rather than binding oxygen, its sixth ligand is either a water molecule or a hydroxide ion [3] (**Figure 4**).

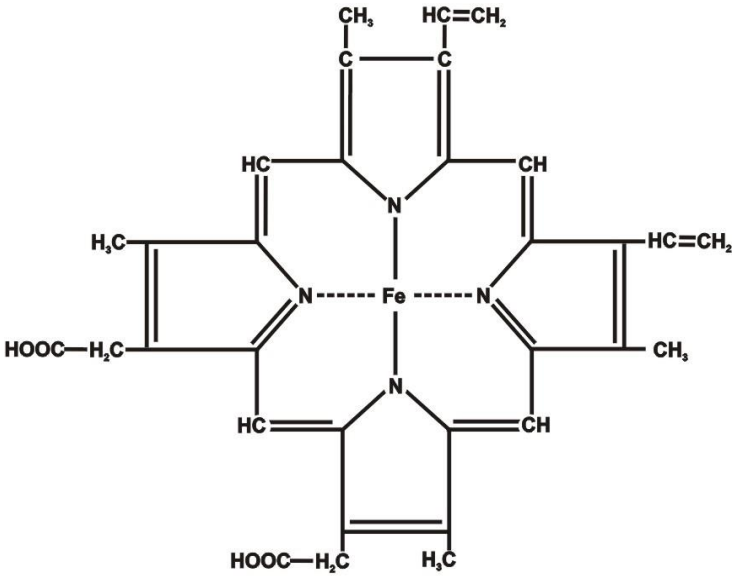

Figure 4. HEME GROUP STRUCTURE AND IRON BONDS

In general, Hb forms an unstable, reversible bond with oxygen. In its oxygen-loaded form it is **Oxyhemoglobin and is bright red.** In the oxygen-unloaded form it is called **Deoxyhemoglobin and is purple-blue.** Oxyhemoglobin is formed during physiological respiration when oxygen binds to Heme component of Hb in RBC. This process occurs in the pulmonary capillaries adjacent to alveoli. The oxygen then travels through the bloodstream and is delivered to all cells, where it is utilized in aerobic glycolysis and in the production of ATP by the process of oxidative phosphorylation [4].

Hemoglobins are present in all kingdoms in nature. All organisms including bacteria, protozoa, and fungi have a protein similar to Hb, whose the known and predicted roles include the reversible binding of gaseous ligands. Since **many of these proteins contain the Heme moiety** (iron in a flat porphyrin support), they are often called Hemoglobins, even if their overall tertiary structure is very different from that of vertebrates. Structure of Hb varies across species: **amino acid sequence of α-globins and β-globins are**

about 50% identical, regardless of which vertebrate species being the source, arguing that these two genes descend from a common ancestor about 450 million years ago, in the ancestral jawed vertebrate [5].

Hemoglobin occurs in all kingdoms of organisms, but it is not the same molecule for all of them. Primitive species such as bacteria, protozoa, algae, and plants often have single-globin Hb. Many nematode worms, mollusks, and crustaceans have very large multi-subunit molecules, much larger than those in vertebrates. In particular, **chimeric Hb** is found in fungi and giant annelids and it may contain both globin and other types of proteins. Plants do not only make oxygen during photosynthesis, but they use it for respiration through the electron-transfer chain in mitochondrion. Some studies show that plants use Hb to bind and transfer oxygen. Some organisms neither have a Hb molecule [5].

Hemoglobin is the most abundant protein in RBC. Catabolism is carried out in the blood by **macrophages that phagocytose senescent erythrocytes.** The Hb molecule is broken up and the iron gets recycled (extravascular hemolysis) or Hb released by the rupture of erythrocytes and immature erythrocytes in the bone marrow (hemolysis). When the porphyrin ring is broken up, the fragments are normally secreted in bile by the liver. Hb deficiency can be caused by decreased amount of Hb molecules, as in anemia.

The **hemoglobinopathies are produced by genes that control the expression of the Hb protein.** Defects in these genes can produce abnormal Hb and anemia. Abnormal Hb appears in one of three basic circumstances: a) structural defects in the Hb molecule; b) diminished production of one of the two subunits of the Hb molecule; c) Abnormal associations of otherwise normal subunits. Other common causes of low Hb include loss of blood, nutritional deficiency, bone marrow troubles, chemotherapy, kidney failure, or abnormal Hb (**Sickle-cell disease**) [5].

Heme, as a ubiquitous iron-containing compound is present in large amounts in many cells and is also inherently dangerous, particularly when it escapes from intracellular sites. Heme greatly amplifies cellular damage arising from activated oxygen. **Hemotoxicity derives from the facility with which this highly hydrophobic compound can enter and cross cell membranes.** Uptake of Heme is required for this synergistic toxicity, and heme hydrophobicity is critical for its entry into cell.

The spontaneous uptake of Heme and the associated amplification of cellular damage are both blocked by **Hemopexin**, which is not the sole factor in plasma that protects against amplified oxidant damage to endothelium. The initial releasing of Heme from free Hb is blocked by complexation with the Hb binding protein, **Haptoglobin.** Furthermore, albumin may also limit the intrusion of extracellular Heme and its pro-oxidant effects. Once within a cell, the Heme can promote oxidative damage either directly, or via the release of iron which can occur either through non-enzymatic oxidative degradation of Heme, or enzymatic, since the Heme oxigenase catalyzes Heme cleavage [5].

2.3. Haptoglobin-Hemoglobin

Haptoglobin (Hp) is a seric tetrameric glycoprotein of acute phase that presents a genetic polymorphism; it is found in abundant concentration in plasma (0.45-3 g/L) [6]. The plasma concentration of Hp increases several times in the event of an inflammatory stimulus such as infection, injury, or malignancy. IL-6 is produced through the activities of the primary cytokines TNFα and IL-1; it is the major inducer of the expression of the protein [6].

Haptoglobin binds with a high affinity to Hb, which is released into the circulation by hemolysis of RBC and by reticulocytes. **The Hp-Hb complex is virtually irreversible.** The capture of Hb by Hp protects against toxic side effects of the oxidative Heme group and directs Hb to macrophages, which are able to internalize Hp-Hb complexes and converts Heme into **Bilirubin. CD163 protein (from macrophage) is the Hb scavenger receptor** [7].

2.4 Hemopexin (Hx)

The human protein Hemopexin (Hx) is a 60 kDa plasma glycoprotein which consists of two homologous, four-blade propeller domains, the amino-terminal and the carboxyl-terminal domains, linked by a linker structure of 20 amino acids. Each domain consists of four repeats of approximately 50 residues, which are suggested to originate from four duplications of an ancestral exon; the two-domain structure of Hx most probably results from gene duplication. **Heme is bound reversibly at the center of Hx in 1:1 ratio** [8].

Hemopexin is the protein with the highest Heme affinity of any known proteins. Human Hx belongs to the **acute phase proteins whose expression can be induced by various cytokines in a context of inflammatory process.** Hx is expressed mainly in the liver but also in the central nervous system (CNS), in peripheral nervous system and in the retina. **The protein transports Heme to low-density lipoprotein receptor-related protein-1 (LRP-1; also named CD91)** [8].

LRP-1 is a receptor for the Heme-Hx complex and is present on several cell types, such as hepatocytes, macrophages, neurons, and syncytiotrophoblasts. Upon binding of Heme-Hx complex to LRP-1, the complex becomes internalized via endocytosis into cells, mainly hepatocytes and macrophages in liver and spleen. Inside the cell, the complex is dissociated by lysosomal activity. Hx can be recycled as an intact molecule to the extracellular milieu [8].

In this manner, the binding of Hx to free Heme limits the amounts of Heme as a catalyst of radical formation in the circulation, makes the essential iron unavailable to invasive microorganisms, contributes to recycling of iron components, and the iron atoms from the Heme enter to the intracellular pool of iron as well.

2.5 Heme oxygenase (HO)

Heme oxygenase is a heme-degrading enzyme that opens the porphyrin ring, producing Biliverdin, carbon monoxide and free iron. Biliverdin reductase catalyses the conversion of Biliverdin to Bilirubin [9, 10]. Endothelial exposure to Heme or Metahemoglobin induces the sequential synthesis of HO-1 followed by Ferritin. **The induction of HO-1 and Ferritin genes is associated with high resistance to injury mediated by oxidant agents.** Three genes encode three isozymes of HO. **HO-1,** identified as a 32.8 kDa stress protein in rat cells but not in human cells, is transcriptionally inducible by a variety of agents, such as Heme, oxidants and cytokines. **HO-2** is constitutively active as metabolizing Heme. The function of **HO-3** is still under investigation in rodents, but is not present in human cells [11].

3. Conclusion

Hemoglobin is one of the most important proteins in the organisms. It is an iron-containing oxygen transport metalloprotein in the red blood cells. Hb in the blood carries oxygen from the lungs to the rest of the body (organs and tissues), where it releases the oxygen. In mammals, the protein includes about 97% of the dry content in erythrocytes. Decreasing of Hb, with or without absolute decrease of red blood cells, leads to anemia. Anemia has different causes, although iron deficiency and its resultant iron deficiency anemia are the most common causes in the world.

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Chapter 6

Transferrin: Indispensable Transport of Iron Atoms in Organisms

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Abstract

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8. References

Abstract

Iron is one the most abundant elements on the Earth, and essential for almost all living organisms. However, iron cannot be accessible for organisms in all environments. The ferric iron solubility is excessively low at physiological pH, and, in aerobic environments, ferrous iron is highly toxic, since in the presence of oxygen leads to the production of reactive oxygen species.

Organisms have developed different ways to obtain iron from the environment. The most important mechanism is the binding of iron to proteins in order to increase its solubility. Primitive organisms carry out the iron capture and transport through organic compounds like citrate, or galate in plants, or siderophores in bacteria. These compounds supply iron to specific receptors on the cell membrane.

Other organisms have evolved Transferrin-like proteins that are similar to Transferrin (Tf) of vertebrates in structure and/or function. In humans, the iron from diet is transported through the enterocyte apical membrane in the intestine by a ferric reductase. Once inside the body, extracellular ferric iron is mainly bound to Tf and in less quantity to Lactoferrin (or Lactotransferrin) (Lf). Tf is the iron transporter that allows iron cellular uptake. Lf is found in milk, saliva, mucosal secretions, and other secretory fluids and is mainly microbicidal and immunomodulator. Both proteins maintain Fe^{3+} in a soluble oxidation state, avoiding the generation of toxic free radicals.

The holo-Transferrin (Tf with two atoms of iron), binds to its specific Transferrin Receptor (TfR) on the cellular surface to be endocytosed. Once inside, the cellular iron is transported to wherever is needed. Iron could be used in mitochondria for the synthesis of several iron proteins. The intracellular iron concentration is regulated by the synthesis of both Ferritin and TfR through the iron-responsive elements (IRE).

At high iron concentration, the body has to defend itself from the toxic effect of iron by increasing the storage capacity of Ferritin and lowering the internalization rate of this element by diminishing the TfR synthesis. On the contrary, when the iron concentration is low, the synthesis of Ferritin diminishes and the TfR synthesis increases, this effect produces a high internalization rate of iron. Cellular responses to iron concentration could be explained by this regulatory mechanism

Key words: iron, transferrin, transferrin receptor.

1. Introduction

Iron has an important role in biochemical processes indispensable for life. **Biological systems have developed proteins and other compounds able to bind iron and transport it to all cells.** A high iron concentration in aerobic conditions is extremely toxic because **iron can produce free hydroxyl radicals via the Fenton reaction** [1], from hydrogen peroxide derived from the cell oxidative metabolism; these radicals are deleterious for most macromolecules.

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

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

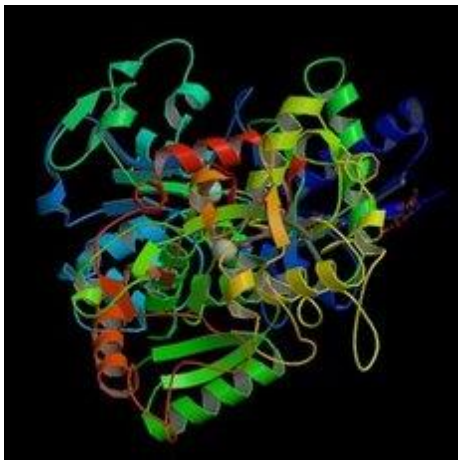
Iron homeostasis is very important for all living organisms; a change in physiological iron concentration has severe consequences in many diseases [2, 3]. Because of the dual effect of iron, being both essential but toxic, its concentration must be controlled at cell level through elaborate systems to ensure adequate iron levels while preventing iron overload [2, 4].

Primitive organisms carry out the iron capture and transport through organic compounds like **citrate, or galate** in plants, or **siderophores** in bacteria. These compounds supply iron to specific receptors on the cell membrane. **Plants obtain this element from soil, using the ferric-chelate reductase enzyme.** This enzyme belongs to the **flavocytochrome family** and its function is to transport electrons through the membrane [5].

Vertebrates transport iron in Transferrin (Tf) (Figure 1), a glycoprotein present in plasma and lymph. Tf binds ferric iron and keeps this element in a nonreactive form in the circulation and extra-vascular fluids, once it is exported from duodenal enterocytes into bloodstream. Iron is transported from the site of absorption or storage to the place where it is required inside or outside the cells [5, 6, 7, 8, 9].

Tf is also involved in iron transport from the liver cells and reticulocytes to proliferating cells throughout the body. The overall purpose of binding iron to Tf is to keep iron soluble in an aqueous medium for distributing this element to the tissues [3, 4].

The human Tf gene is located at chromosome 3q21-q25. The gene encodes a glycoprotein of almost 80 kDa, **synthesized in hepatocytes and secreted to the plasma** [3, 5].



http://www.cs.stedwards.edu/chem/Chemistry/CHEM43/CHEM43/Projects04/Transferrin/structure.htm

Figure 1. TRANSFERRIN MOLECULE

Transferrin is composed of a single bilobal chain with a combination of alpha helix and beta-sheet structures containing N- and C-lobes, each with two domains connected by a hinge. This creates a cleft that contains the iron-binding domain. The amino acids that bind ferric iron in both lobes are: two tyrosines, one aspartate and one histidine. Iron binding and iron release are coordinated by a conformational change

2. Transferrin Structure and Function

The affinity constant of Tf for ferric iron (Fe^{3+}) is about 10^{-20} M. Tf is freely soluble in water and **is able to bind two atoms of iron.** Iron-saturated Tf (holo-transferrin) (**holoTf**) is much more stable to denaturation than non-saturated Tf (apotransferrin) (**apoTf**). HoloTf presents a salmon-pink colour in the presence of carbonate, a synergic anion that participates directly in the iron binding to Tf [9, 10].

The iron necessity for cell proliferation is related to the activity of the **ribonucleotide reductase** in the synthesis of DNA in the majority of the organisms**. This enzyme converts ribonucleotides in deoxy-ribonucleotides. Besides, iron is involved in energy production, breathing, metabolism, and binding to several macromolecules** [11, 12].

The **mammalian Tf molecule** is about 80 kDa with two carbohydrate chains that bind to the N-terminal region [13]. It **has two lobes** (one at N-terminal and other at C-terminal), **probably as a result of an event of duplication of genes** [9, 10]. Each lobe has the capacity to bind an iron atom with different affinity, to keep it in its non-reactive and soluble form. The C-terminal binding site is the most avid for iron [6, 9, 10, 14], as well as this site is less susceptible to the loss of iron at range of slightly acidic pH. Tyrosine and histidine residues of the side chains of amino acids are involved in the iron binding. The specific Transferrin Receptor (TfR) internalizes the iron inside the cells [6, 9].

The half-life of Tf is about 8-12 days. Its concentration in plasma is between 2 and 4 g/L in healthy adults and children, and it does not change with sex or age. In normal iron condition, **Tf is saturated by approximately 30%,** therefore, the molecule is always avid for iron and it has the capacity to bind it [13, 14]. Tf and other similar proteins were developed in the evolutionary process of the organisms and they were **conserved due to the importance of the iron transport and the necessity of binding this element in order to avoid toxicity.**

3. Transferrin-like proteins

Transferrin-like proteins are similar to Tf in structure and/or function. They have been found in various organisms, e.g. the alga resistant to high salt concentration ***Dunaliella salina,*** which has developed a special mechanism for iron acquisition and regulation. **High salinity and low iron are necessary for a specific protein binds iron with high affinity in order to adapt to this environment. The Tf-like protein of these algae has a similar affinity for iron as the common Tf** [5, 15]. In general, **seaweeds obtain iron from soil through a ferric-reductase enzyme,** similar as it was observed in plants. **These Tf-like proteins have three lobes and the iron binding requires the presence of a carbonate ion** [5]; it is present on the membrane surface. The iron regulation is carried out by the iron accumulation inside the cell, avoiding the binding and entrance of more iron atoms. The iron captured is stored in acidic vacuolar structures [16].

Cryptococcus neoformans [17], **a fungus** that produces meningitis in AIDS patients, requires iron to survive. This organism produces a Tf-like protein of about 80 kDa of molecular mass, it is similar to Tf from mammals and is recognized by an anti-ovotransferrin antibody; this indicates some structural or sequence similarity between these proteins. Recently, other iron binding molecules have been reported in this fungus, like **siderophores,** which capture iron from host iron-proteins, and the capture of iron bound on the membrane by **the permease-ferroxidase system** [18].

In the enteric bacterium that causes the typhoid fever, ***Salmonella enterica*** **serovar Typhi,** a cell wall protein was recognized by an anti-Tf antibody, so this protein could be participating in the iron acquisition system [19]. Unfortunately, this protein has not been characterized yet.

Other Tf-like proteins have been observed in invertebrates such as in the ***Cancer magister*** **crabs** [20] **and in tarantulas**; the molecular masses of these proteins are of 150 and 80-100 kDa, respectively. In **the tunicate *Pyura stolonifera*** the Tf-like protein is of 40 kDa of molecular mass with only one iron binding site [5, 21]. Additional information is needed to know if these proteins are involved in the mechanism of iron acquisition.

In insects, Tf-like proteins show some homology with human Tf, such as Tf from the insect of the bean, ***Riptortus clavatus***, which Tf-like protein of 75 kDa is 25% homologous with human Tf. This protein is

indispensable for the larva development [22] and it is 43% homologous to Tf-like proteins from other insects such as the butterfly ***Manduca sexta*** (77 kDa), the transmitting mosquito of yellow fever and dengue viruses, ***Aedes aegypti*** (66 kDa), and the cockroach ***Bablerus discoidalis***. All of these proteins bind iron in synergy with a bicarbonate anion, they present tyrosyl residues involved in the iron binding site, and they release iron depending on pH [5].

Interestingly, in ***Protaetia brevitarsis*****, scarab of humidity,** the involvement of a Tf-like protein was observed in defense mechanisms and response to stress. **RNA interference technology was used to diminish the Tf concentration; this caused an increase in free iron and H_2O_2 levels in the hemolymph, with the induction of cellular death by apoptosis** [23]. In the same way, when the red ant ***Solenopsis invicta*** was infected with a specific pathogen fungus, and ***Glossina morsitans morsitans*** (tsetse fly) was infected with pathogenic bacteria, the Tf genes were induced by stress response, besides the Tf function of iron transportation to the developing embryos [21, 23, 24, 25].

In the flies *Sarcophaga peregrina* and ***Drosophila melanogaster***, the Tf-like proteins have been purified from hemolymph, both proteins have a molecular mass of 65 kDa with a homology of 36% and only one iron binding site in the N-terminal region. **Same as occurs in the insects mentioned above, these Tf-like proteins increase their concentration during an infection process and are considered as acute phase proteins, which act as antioxidants controlling the iron level** [25].

Tf-like proteins from insects show a deep resemblance in the iron binding site with those of vertebrates, although they present only one functional site [5]. Based on this evidence, it was proposed that Tfs from vertebrates are related with the sequence present in **a common ancestor to both, arthropods and vertebrates.** Some eukaryotes have analog iron binding proteins with one or two lobes that are very similar to Tf from mammals. Hence, it has been proposed that Tf was evolved at the same time that the functional kidneys, increasing the size of the protein to avoid lost by filtration [6]. In this way, **Tfs are the consequence of a gene duplication event in the common ancestor,** being this the origin of the family of Transferrin proteins [5] (**Figure 2**).

The amino acid comparative analysis of the Tf-like protein from *Bablerus discoidalis* with that of *Manduca sexta* and with human Tf revealed several interesting findings: 121 residues are conserved, mainly the cysteine residues at 19 position; three of the four amino acids (Asp63, Tyr95, Tyr188, and His249) involved in the iron binding are conserved; and the anion-binding amino acids that are present in vertebrate Tfs (Arg124 and Arg456) are switched by a neutral residue.

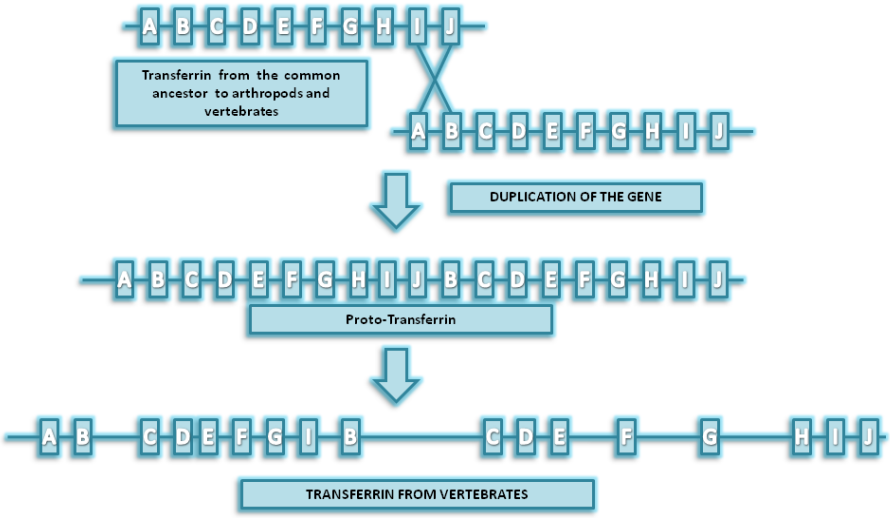


Figure 2. TRANSFERRIN MOLECULES

A common ancestor gene duplicated as a result of a recombination process, generating a duplicated ancestor (proto-Tf). The Tf molecules from insect and vertebrates conserve the sequence present in the last common ancestor of vertebrates and arthropods; these are observed in the gene and in the molecular mass of the protein.

In primitive vertebrates such as **the lamprey *Geotria australis,*** the Tf-like protein is built up by four 78 kDa subunits with two iron-binding sites per subunit. The carp ***Tinca tinca*** and the frog ***Xenopus*** spp. have also shown to possess the capacity to synthesize Tf-like molecules with molecular mass of 78 and 85 kDa, respectively.

Pacifasacus leniusculus, **a freshwater fish,** presents the **pacifastin** protein [7], **which is functional and structurally related with Tf.** It was purified as a protease inhibitor, formed by two subunits bound each other covalently: one heavy chain of 105 kDa, trilobulated, with one or two iron-binding functional sites and the light chain of 44 kDa with similar structure of protease inhibitors of low molecular mass. Two lobes of the heavy chain (1 y 3), are similar to the N-terminal region of Tf and the other one (2) is similar to the Tf C-terminal region [5].

4. Transferrin Synthesis

Tf is mainly synthesized in the liver and in a little amount in testis, spleen, kidney, brain, intestine, lung, and bone marrow [6, 9, 13]. In the different tissues, Tf gene expression has been quantified, for example, in rat there are 6,500 mRNA molecules/hepatocyte, 100 molecules/brain and testis cell, and only 12 molecules in

other tissues [22, 25]. **The expression of Tf gene is completely different in fetal development than in adults.** In this period the expression rate is higher in lung, kidney, intestine, and liver, while in the brain there is almost no expression [22]. **The Tf gene synthesis and expression are regulated by several mechanisms and its regulation depends on the specific tissue** [13].

The scarcity of iron induces the Tf gene expression in the liver of human beings, rats, mice, and chickens with the consequent increase in protein quantity [25]. Nevertheless, **in humans with a bad nutrition or with some inflammation process, the iron scarce produces the overexpression of the Tf gene, but the synthesis of the protein diminishes.** This was observed in severe undernourishment cases associated with a high percentage of mortality due to iron insufficient in diet or by infections [13, 14].

In the same way, **in rats** [27] **and lambs** [28] it has been observed that **both glucocorticoids and estrogen stimulate the Tf synthesis in the mammary gland during breast feeding,** although Tf is depleted of iron. This is due to that Tf is released from acid vesicles to the alveolar lumen by **transcytosis,** and not by the specific synthesis in this gland.

The iron regulation is determined by the amount of iron that enters to the human body in enterocytes. This amount could be increased under conditions of iron scarce or anemia, and could be reduced in iron overload condition. In the last years, several proteins involved in iron transport have been studied such as: Tf, TfR, Divalent Metal Transporter-1 (DMT-1), Ferritin, **Ferroportin, Hephaestin,** and Iron-Regulated Proteins (**IRPs**), among others.

5. The Transferrin Cycle

Tf is internalized into the cells forming a complex with the TfR [11]; internalization occurs **in clathrin-coated vesicles** [4, 6, 27, 28], formed in cell membrane regions rich in receptors which are invaginated to form cover endosomes. Once the vesicle is released from the membrane, **clathrin is removed and the vesicle is directed to the network of membrane compartments such as endosomes and tubular structures.**

The endosomes are acidified by the proton efflux; the pH changes to $4.3 \leq 6.5$ and there are conformational changes in both Tf and TfR. Then **the iron is released and the apoTf-TfR complex is taken back to the cellular membrane** [26, 29, 30], **where apoTf is released and is able to bind new ferric ions.** Iron-saturated transferrin (holoTf) is bound to the TfR in the cellular surface [31]. The recycling of the vesicle to the cellular surface takes just a few minutes (**Figure 3**).

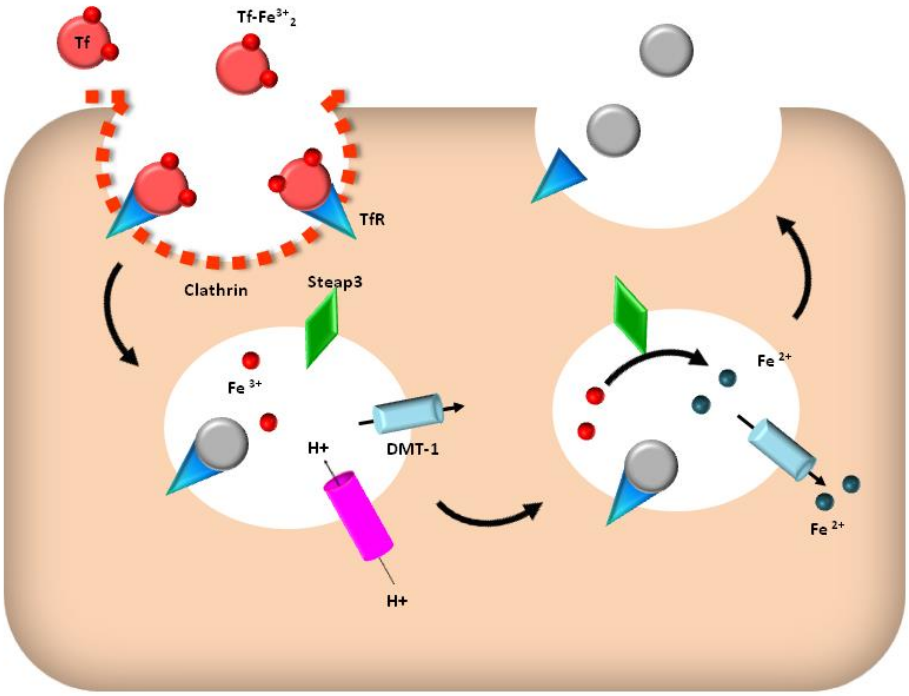


Figure 3. TRANSFERRIN CYCLE

ApoTransferrin binds two ferric ions, a typical salmon-pink color is acquired and it is called holoTransferrin (holoTf). Tf receptor (TfR) recognizes and binds holoTf, the complex is internalized inside a clathrin-coated vesicle. Inside the endosome the pH is acidified and promotes the ferric iron release by its reduction to ferrous iron that allows it to be transported by the Divalent Metals Transporting-1 (DMT-1) protein, towards the cytoplasm. Once in cytoplasm, it is incorporated to proteins and detected by Iron Regulating Proteins (IRPs).

Iron in the N-terminal region of Tf is released in milliseconds and that in C-terminal in hundred of seconds. **Most of Tf is found in biologic fluids** [25, 32]. This cycle does not explain how iron enters to the cytoplasm although this process begins to be revealed (**Figure 4**).

First, ferric iron (Fe^{3+}) is reduced to ferrous iron (Fe^{2+}), by a transmembranal ferrireductase, Steap3 [2, 8, 30]. The interaction between Steap3 and Fe^{3+} produces a conformational change in Steap3 that generates an electron flux through the membrane [2, 6]. **DMT-1,** a member of the Natural Resistance Macrophage-Associated Protein-2 family (**Nramp2**) **transports Fe^{2+}** [6, 8, 31, 33]. The iron transportation depends on temperature, energy, and pH. Most of the activity necessary for iron release inside endosomes was observed at pH 5.5 [8]. Thus, **Nramp2/DMT-1 is found mainly in late endosomes and lysosomes but not in early endosomes.** This is evidence that this protein participates in Fe^{2+} iron transportation to cytoplasm

[34]. **The iron transportation to cytoplasm is essential for the cellular metabolism of iron.** DMT-1 forms a channel that is permeable to ferrous iron **with different affinity degree.** The malfunction of the channel is related with mucolipidosis type IV, **characterized by motor weakness, mental retardation, retinal degeneration, and iron deficiency anemia [35].**

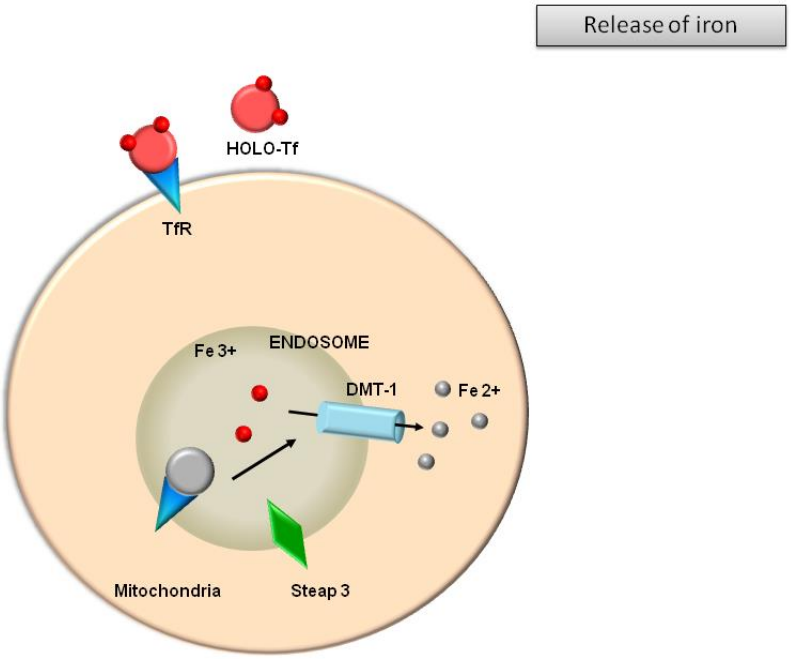


Figure 4. IRON TRANSPORTATION FROM ENDOSOME TO CYTOPLASM

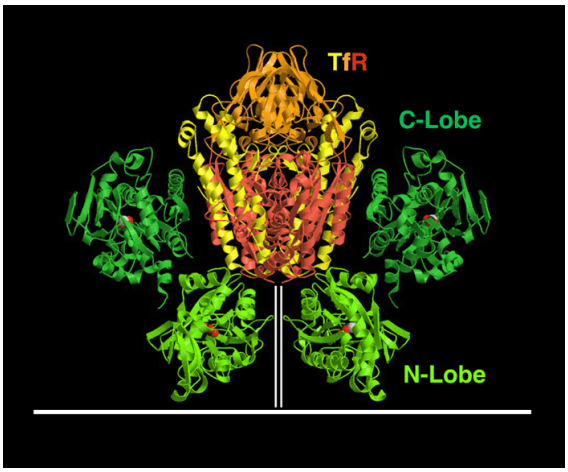
After endosome acidification, iron is released into the cytoplasm through the Divalent Metals Transporter protein (DMT1); ferric iron is oxidized to ferrous iron.

Both STEAP3 and DMT-1 were found through positional cloning of genes altered in rodents carrying spontaneous mutations related with iron transport [6, 30, 31]. These genes are important for human health, because its presence or absence is related with cancerous process or other diseases associated with anemia [2]. The mechanism of regulation is through DMT-1 and is dependent on iron, similar to the mechanism of RTf1 [31].

6. Transferrin Receptor 1 and 2

The TfR1 (**Figure 5**) is a dimeric glycoprotein formed by two subunits of approximately 90 kDa of molecular mass. At the beginning it is synthesized as a peptide of 79 kDa, and then carbohydrates are added in

endoplasmic reticulum. Post-translational modifications such as acylation and phosphorylation of serine and threonine occur in the Golgi apparatus.



http://walz.med.harvard.edu/Research/Iron_Transport/TFrTF.php

Figure 5. TRANSFERRIN RECEPTOR-TRANSFERRIN

The molecules of Tf bind laterally to a dimeric receptor between a part of the receptor and the cytoplasmic membrane. Red: TfR, protease-like domain; orange: Apical region; yellow: TfR helix; light green: N-terminal region of Tf; dark green: C-terminal region. The receptor tail and the membrane surface are represented with white lines.

Human TfR1 was purified by Seligman et al [36] and later its size and stoichiometric union with Tf were determined [29]. The cellular function of this receptor and its role in iron capture were studied in cellular cultures. **Tf with two iron atoms preferentially binds to TfR1 at physiological pH.** Receptor could attach monoferric and apo-transferrin forms with less affinity.

TfR1 is expressed at low levels in several tissues and few are the cells that strikingly depend on Tf to obtain iron. It was observed that the interruption of the TfR1 gene in mice has no effect in the development of tissues except in erythroid and neuroepithelial cells that need TfR1 for differentiation [6, 37, 38].

Recently, it was identified a second TfR, the **Transferrin Receptor 2 (TfR2), similar to TfR1, but the affinity of TfR2 for Tf is 25 times lower than the TfR1 and it is expressed just in liver and erythroid cells, while TfR1 is observed in a great variety of cells,** as was mentioned before. So, the expression of TfR2 is not related with changes in iron levels [9]. Lately, this receptor is related with hepcidin regulation in response to iron concentration [39].

6.1 Regulation of the Transferrin Receptor 1

The classic transferrin receptor, TfR1, is found in high concentration in dividing cells, in activated lymphocytes, and in precursors of erythroid cells [11]. In these cells, the iron cellular metabolism is regulated by iron itself, by Ferritin, and by the number of TfR1 molecules. All of them coordinately control the entry of this metal. For example, **when iron is in high concentration, cells synthesize Ferritin to store the abundant iron.** At the same time, **the stability of the mRNA of the TfR1 diminishes, so the synthesis of the receptor and the iron entry are reduced** [9, 40].

Inversely, when iron level is low, the synthesis of Ferritin diminishes and the receptor mRNA is stabilized and more quantity is synthesized, then the iron entry is high [40]. **The levels of the complex Tf-RTf1 are the limiting factor in the regulation of the iron internalization** [8]. The iron-response elements, **IREs, are responsible of this process** (**Figure 6**). These are found in non-translated regions of mRNA and they are structures conserved of stem and loop of mRNA, where the iron-response proteins (**IRPs**) bind.

The Ferritin mRNA contains the IREs in the 5'-non-translated region. **When iron concentration is too low, IRPs binds to IRE in the mRNA of Ferritin and avoids its translation.** The presence of several IREs in the 3' and 5'-non-translated regions of the mRNA of TfR1 stabilize it. These two activities are the base for the iron homeostasis in most of the cells [41, 42].

The importance of the IRE-IRP regulation system was demonstrated in hemochromatosis [43]. This disease is characterized by excessive iron absorption, that produces the pathological increase of this metal, because the low level of Hepcidin (protein that regulates Ferroportin), that produces iron accumulation (see section 7.2. Macrophages).

7. Iron transporters and their relationship with iron and Transferrin

An important aspect of iron metabolism is that it is handled in different forms in different types of cells [4]. For example, in duodenal enterocytes the iron is captured passing from the external medium to inside the cell, and afterwards is released from this cell to the external medium, in order to be transported to the whole organism. This function is carried out by iron transporting proteins that are much related with the Tf function. **During the production of erythrocytes, a great amount of iron is needed to bind Tf, and most of this iron is used for the hemoglobin synthesis.** In these cells, the function of exportation or release of iron is very necessary.

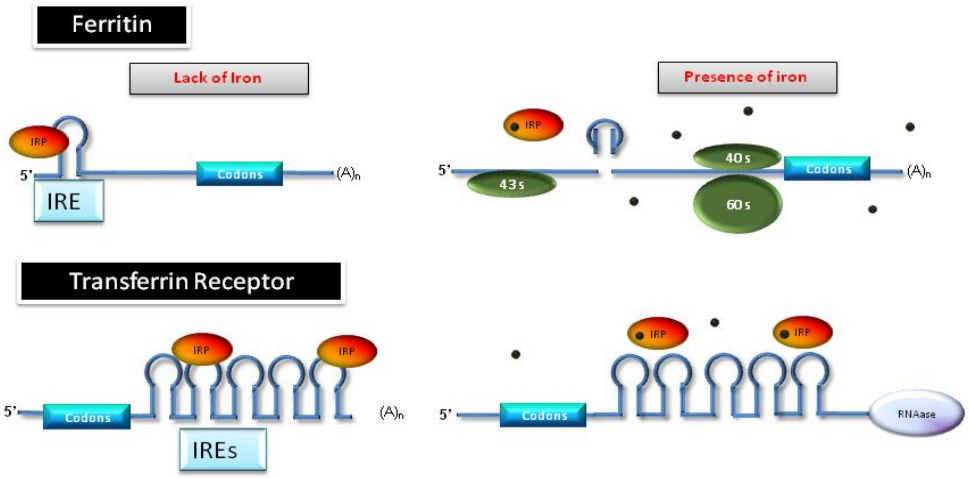


Figure 6. REGULATION OF THE IRE-IRP SYSTEM

When iron concentration is low, IRPs binds to IRE in Ferritin mRNA and inhibits the translation; the TfR mRNA is stabilized by the binding of IRP to IRE on the 3' non-translated region of mRNA of the receptor, synthesized in great amount, in this way the iron entry is increased. When iron is in high concentration, cells use mRNA to synthesize more Ferritin for iron storing. At the same time, the stability of the mRNA of TfR1 diminishes and so the synthesis. Then, the iron internalization is reduced. IRPs act as regulators in iron homeostasis, by controlling genes involved in iron metabolism.

7.1. Duodenal enterocytes

In intestine epithelial cells, Steap3 reductase reduces ferric to ferrous iron in order to be internalized in the apical membrane of the enterocyte through DMT1. Later, the iron inside the cell goes towards the basal membrane to reach the extracellular medium through the ferroportin [43], and a transmembranal ferroxidase (hephaestin) produces the iron oxidation from Fe^{2+} to Fe^{3+}. It creates a gradient of concentration through the membrane facilitating in consequence its exit. **At low iron concentration, the mRNA translation of DMT1 and the ferroportin are increased, the opposite happens at high iron concentration** [44] (**Figure 7**).

Recently, **IREG1,** a protein involved in iron absorption from duodenal membrane was isolated [45]. This protein was **localized in the basolateral membrane from epithelial cells and facilitates the iron exit through ferroxidase.** The expression of IREG1 in mucosa is important for the control of iron absorption from diet and homeostasis in the human body. It seems that IREG1 participates in iron active transport in patients with hereditable hemochromatosis.

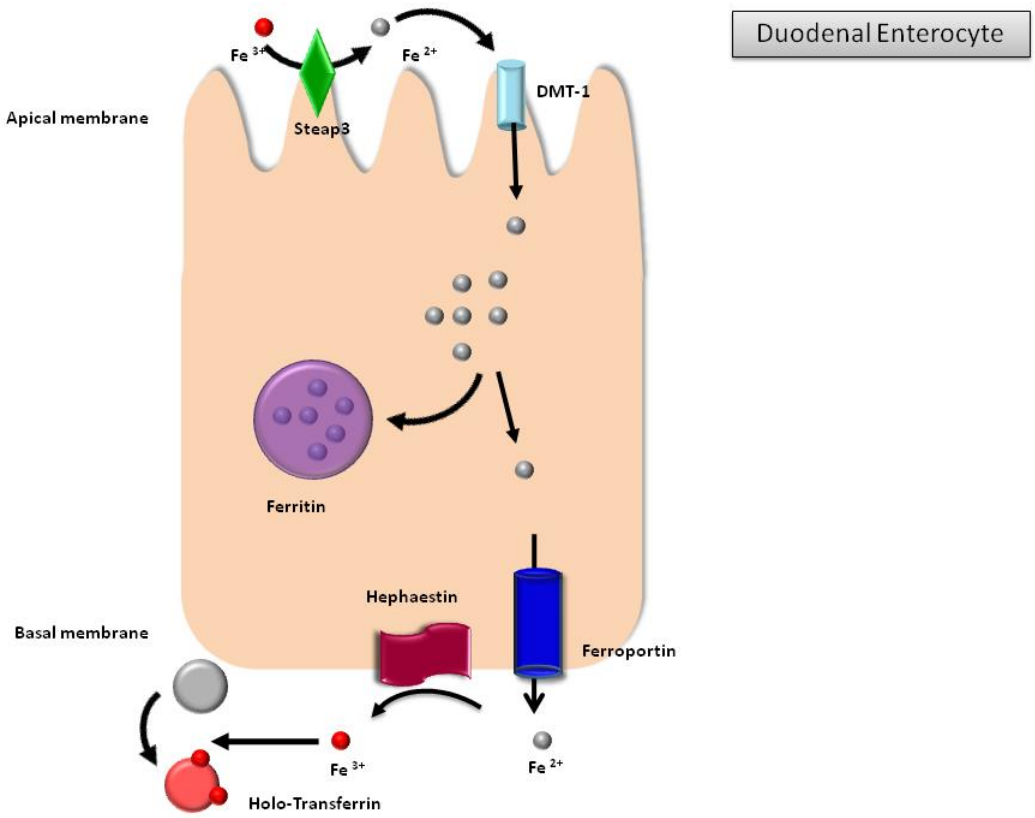


Figure 7. ENTEROCYTES OBTAIN IRON FROM DIET

The Fe^{3+} is reduced by the ferrireductase (Steap3) in the apical membrane and is transported by DMT1 inside the cell. The iron could be stored in Ferritin and the excess passed through the basal membrane to plasma. Ferroportin1 and hephaestin take out iron from cells, where apoTf binds it.

7.2 Macrophages

Macrophages are very important cells for recycling iron from senescent erythrocytes. In these cells other proteins are related with the mechanism of obtaining, storing, and releasing iron. The study of these mechanisms allows a better understanding of the molecular process used for maintaining the homeostasis of this indispensable metal.

Among all these proteins, it was found that **Hepcidin is considered the most important iron regulator until now.** It is a little peptide of 25 amino acids, first identified in plasma and urine, as an antimicrobial peptide [5, 46]. It acts in absorption and release of iron in macrophages. **In high iron concentration, the Hepcidin is bound to Ferroportin in the basal membrane; it produces the internalization and the degradation of Ferroportin, avoiding the exit of iron. This produces a hypoferrhemia [46] very important to avoid the colonization of parasites.** The production of Hepcidin

increases in the iron presence and/or in some inflammatory processes and diminishes when iron is lacking, this produces the increase of Tf, TfR1 and TfR2, and other proteins that inhibit Hepcidin to return to normal iron balance [5, 39, 46].

In all these iron transporting proteins, the regulation is mediated by iron concentration [48]. The expression of DMT1, the duodenal Ferrireductase, Ferroportin and Hephaestin vary according to the kind of cell (**Figure 8**) [48].

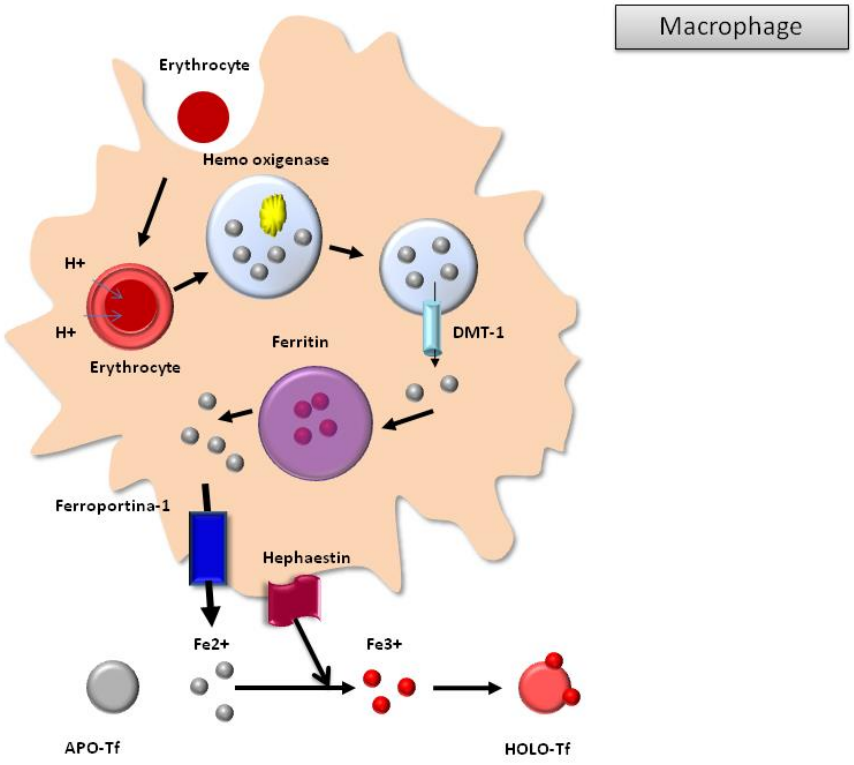


Figure 8. MACROPHAGES CARRY OUT THE IRON RECYCLING
The senescent erythrocytes are ingested and processed by macrophages, hemoglobin is digested inside the phagolysosomal compartment, where the iron is released from the hemo group and transported inside the cell. This process involves Ferroportin 1.

7.3. Hepatocytes

Hepatocytes are the cells used mainly for iron storing in mammals [4]. High Ferritin concentration is normal in these cells, where the excess of iron transported by the cycle of Tf is captured. **These cells present the alternative mechanism to capture the iron not bound to Tf.** Once inside the cell, iron obtained from the two mechanisms is found in Ferritin.

According to the necessity of iron in the organism, the hepatocytes promote the iron exportation by the binding to Tf, and then it can return to circulation (**Figure 9**).

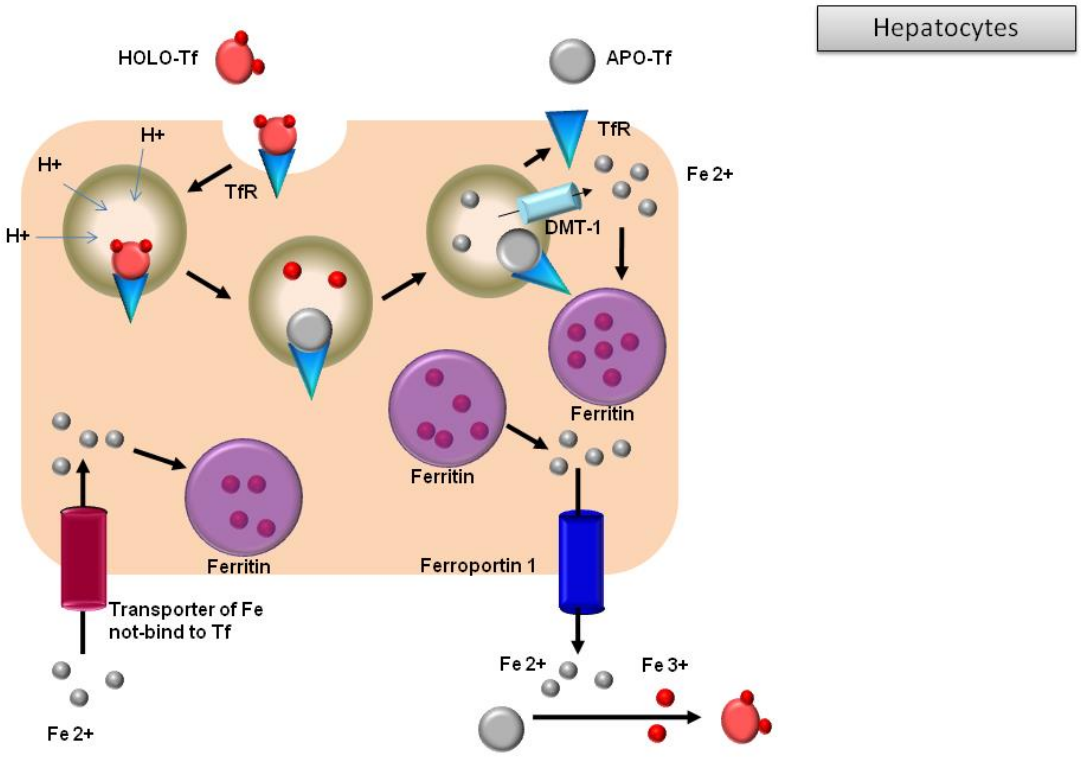


Figure 9. HEPATOCYTES CAN TAKE UP IRON THROUGH TWO DIFFERENT VIAS, BY THE CYCLE OF TRANSFERRIN AND BY THE IRON NOT BOUND TO TRANSFERRIN

Iron is stored in Ferritin, but when it is required it can be released using Ferroportin 1.

7.4. Erythroid cells

The iron transported by Tf is captured by its receptor (TfR1 and TfR2), and released inside endosomes. The DMT1 protein allows the iron transport to cytoplasm and mitochondria, where the majority of molecules are incorporated to form part of Hemoglobin [4] (**Figure 10**).

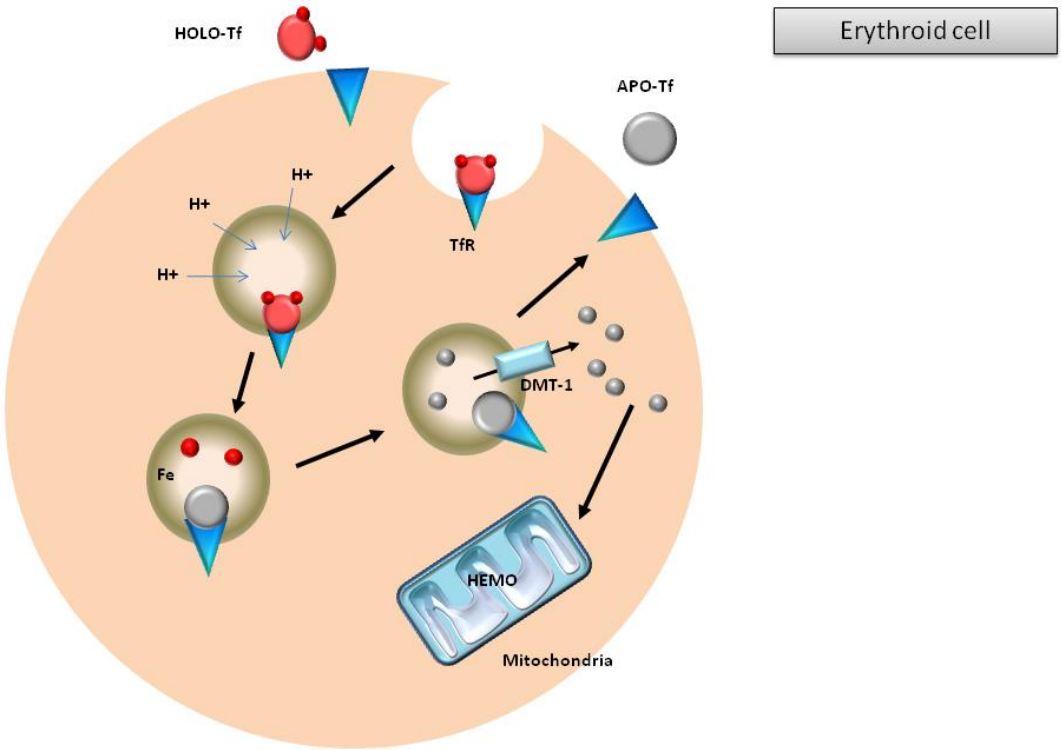


Figure 10. ERYTHROID CELL

They obtain iron from the Tf cycle and use it for Hemoglobin synthesis; the erythrocyte precursors do not export the iron.

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Chapter 7

Lactoferrin: A protein that sequesters iron on mucosal surfaces

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Abstract

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Abstract

In previous chapters of this book, proteins that bind, store, and transport iron were described. The present chapter briefly describes the structure, function, and applications of a Transferrin family member called Lactoferrin or Lactotransferrin (Lf).

Surely, after reviewing this chapter, you will appreciate how interesting this protein is. Although human and bovine lactoferrin share a high homology and identity, compared to other Transferrin family members, Lf has very different functions and properties. Small differences in its structure and three-dimensional shape confer amazing abilities to Lf, particularly its multifunctionality and applicability, which are features that are not found in other Transferrin family members.

In fact, Lf is a prominent component in the first line of mammalian host defense, mainly in the mucosa. Although it has been assumed that Lf has particular functions, in different physiological environments or biological locations, these functions are actually quite different, which is a consequence of the ability of this protein to bind to various types of biological molecules and cells.

Key words: iron, lactoferrin, proteins, transferrin, multifunctional

1. Introduction

Lactoferrin (Lf) is a non-heme, iron-binding glycoprotein that consists of a single polypeptide chain of 703 amino acid residues with a molecular weight of 80 kDa. **Lf belongs to the Transferrin family** (iron-containing proteins), which includes the major iron-transporting protein in the blood (**Transferrin; Tf**), an egg-white protein (**Ovotransferrin**), a melanocyte protein (**Melanotransferrin**) and a recently identified **Carbonic anhydrase inhibitor** [1].

Lf is a prominent component in the first line of mammalian host defense, mainly in the mucosa. Additionally, Lf is the major iron-binding protein in human milk and is important for iron absorption in the human body [2,3,4].

2. Synthesis and localization of lactoferrin

Lf is synthesized by exocrine glands and is secreted by epithelial cells, secondary granules of neutrophils and some acinar cells [5,6]. Within mucosal surfaces, Lf is widely distributed in mammalian milk and other exocrine secretions, including tears, saliva, intestinal mucus, nasal and genital secretions, and the secondary granules of neutrophils. Furthermore, lower concentrations Lf are found in the blood [7,8].

With regard to its iron-binding properties, **Lf differs from serum Tf in its higher iron-binding affinity, its unique ability to retain iron over a broad pH range, and its distinct tissue distribution relative to other Tfs**. All of these differences contribute to the unique functional properties of Lf. Based on its ability to retain and release iron, Lf can exist in two states, the iron-containing and iron-free forms. The iron-containing form, known as **hololactoferrin (holoLf),** is conformationally more rigid and stable compared with the iron-free form, **apolactoferrin (apoLf)** [9].

2.1. Why is Lf localized in the secondary granules of neutrophils?

Lf is contained in the secondary granules of neutrophils. It is released at the site of microbial invasion, and after binding iron (Fe), the complex is bound and ingested by macrophages. Iron is essential for both pathogenic microbes and their hosts. In the human body, bacterial infections are often associated with a reduction in circulating iron, a calibrated host defense mechanism that deprives microorganisms of a nutrient required for their growth and virulence. This iron reduction is known as **hypoferremia of infection** [8]. As pathogens are unable to grow in the iron-deficient environment, they die due to iron deprivation, indicating that Lf is **microbioestatic** [10,11].

Two factors contribute to lactoferrin's efficiency in iron sequestration in the reticuloendothelial system. First, due to its higher affinity for iron at a pH lower than physiological levels, **Lf binds more iron than transferrin at inflammation sites (sites of low pH).** Second, **lactoferrin receptors** (**LfRs**), **which are**

present on the surface of macrophages, are not expressed on erythroid precursors. During inflammation, activated macrophages exhibit an increase in the LfRs concentration on their surface, which further increases iron internalization and thereby reduces the iron available for microorganisms and erythroid precursors [8].

Lf synthesis is regulated in each tissue and is controlled by hormones [12]. In humans, Lf is secreted in the iron-free form (ApoLf) from mammary glands epithelial cells, tears, saliva, and the exocrine secretions of mucosal surfaces located in the lungs, reproductive tract, and pancreatic and gastric juices [13]. In addition, Lf participates in the primary immune response in newborns [12].

Lf is found in very small amounts in the plasma, and it is predominantly derived from neutrophils. Lf concentrations can be low under normal conditions but elevated during inflammation due to the degranulation of neutrophils, which releases it [13].

Lf concentrations in humans range (from highest to lowest) from 7-15 mg/ml in colostrum, 2.2 mg/ml in tears, 1.2 mg/ml in mature milk, 0.44-1.92 mg/ml in seminal fluid, 62.9-218 µg/mg of protein in vaginal mucous, 28.7-41.7 µg/mg in bronchial mucous to 2-32 µg/mg in amniotic fluid. Under normal conditions, the Lf concentration in liquid plasma is relatively low (0.4-2.0 mg/L); however, **in patients with sepsis, neutrophils are activated and degranulated, resulting in the secretion of significant levels of Lf** (~ 200 mg/L) mainly in the iron-free form (apoLf) into the bloodstream [7].

3. Lactoferrin structure, iron uptake and release mechanisms

Lf is a monomeric glycoprotein of approximately 703 amino acid residues that has a Mr of 78-80 kDa [14], Lfs show a 60% sequence homology with serum Tf. **The polypeptide chain is folded in two lobes with approximately 40% sequence homology, which is indicative of ancestral gene duplication that occurred millions of years ago.** As a result of this process, the original gene, which encodes a 40-kDa protein, was copied and reproduced, resulting in two domains and a family of proteins with Mr of 80 kDa [15,16]. Each lobe is folded into two domains separated by a cleft, where each iron-binding site is located. In all cases, the ferric iron is ligated to an aspartic acid, two tyrosines and a histidine, with two oxygens atoms that form a synergically bound carbonate ion, without which Lf cannot bind iron [15].

Iron binding and release are associated with conformational changes in the protein. When Lf is in the iron-free form (apoLf), the interdomain cleft adopts an “open” configuration, and the binding of an iron and carbonate ion causes the cleft to close and become locked in this configuration. Thus, the open apo form is more susceptible to denaturation and proteolytic digestion than the Fe-bound (holo) form. Iron is released by lowering the pH, but in lactoferrin, the degree of stabilization of the lobe structure is greater than in Transferrin. Consequently, iron release does not occur until a pH of 3 is reached, whereas iron release occurs at a pH of 5.5 in Tf [14,15]. This difference in pH sensitivity explains why Tf, but not Lf, can donate iron to cells via receptor-mediated endocytosis.

As previously mentioned, Lf exists in Fe-free (apoLf) and Fe-saturated (holoLf) forms. These molecules have different 3D conformations, which is an important, distinguishing characteristic. **HoloLf is more resistant to degradation and temperature than apoLf.** The main differences between apoLf and holoLf are located in the N lobe [17]. In apoLf, the N lobe adopts an open conformation (an angle of 53° between subdomains N1 and N2), while the C lobe adopts a closed conformation (C1 and C2 subdomains are nearby), providing resistance against heat denaturation and proteolysis.

The closed conformation of holoLf is lost when the structure is destabilized, such as when Lf is recognized and bound by a bacterial receptor or host cell receptor (the same applies to Tf), when it is located in a strongly acidic media (less than 4.0), or when there is protonation of the carbonate ion (the closed structure is destabilized and Fe is released). According to the conformational structures, the differences between apoLf and holoLf are simple movements in the protein domains, and **the shape adopted by apoLf confers its ability to interact with a variety of molecules located in cells or biological systems** [18,19].

The amino acid sequence of Lf has been studied in Lf from different sources. The first sequence was studied in Lf from breast milk, and cDNA generated from myeloid cells and the mammary gland. These sequences were virtually identical. However, the crystallographic structures of different human Lfs suggest that there are three arginine residues (GRRRS), not four (GRRRRS), present in the N-terminus of the protein. This discrepancy is due to the flexibility located in the N-terminal region of the protein [20].

4. Isoforms of Lactoferrin

Lf exists in three isoforms: Lf-β and-λ Lf, which have RNase activity, and Lf-α, which has no RNAse activity. The three isoforms are found in breast milk and in neutrophils [21,22]. They share the same physical, chemical and antigenic properties but differ in their functional properties. Isoforms with RNase activity cannot bind Fe, whereas the Fe-binding isoform does not exhibit RNase activity. These isoform differences partially explain the diversity of the different functions attributed to Lf**.**

In addition to full-length Lf, alternative splicing can also produce a truncated form, called delta lactoferrin. Delta Lactoferrin (ΔLf), an intracellular lactoferrin isoform can act as a transcription factor. Its expression is down regulated in cancer, also high expression level of its transcripts has been found in normal tissue and was reported to be absent from their malignant counterparts. Several studies have indicated that delta-Lf acts as a transcription factor inside cells and that it is involved in the regulation of the expression of specific genes [23]. Clearly, taken together these data indicate that Lf can play a role in cancer, and this area of research deserves more attention in the future [24].

Lf from human milk has three potential sites for glycosylation at asparagines (Asn) 137, 478 and 623. Only two are essential and important, with one located at the C-terminus and the other at the N-

terminus, but the degree of glycosylation varies (hence the protein molecular mass ranges from 76-80 kDa). However, the glycosylated residues are typically present in Lf [25]. Mouse Lf only has one site (Asn476), human Lf has four (Asn233, Asn368, Asn476 and Asn545), and Lf from cow, goats and sheep has five (Asn233, Asn281, Asn368, Asn476 and Asn545) sites for glycosylation [18,19].

Do these sites have some impact on the structure and function of Lf? This question is interesting, and it cannot be answered with currently available data. It is known that glycosylation sites are exposed on the protein surface; however, Baker *et al.* noted that the release of holoLf Fe during its interaction with other molecules (DNA and LPS) is not affected by the lost carbohydrates. Thus, these sites do not contribute to the bactericidal activity of Lf, at least in Gram-negative bacteria, which contain LPS on outer membrane [18,19].

The glycans found in Lf are sugars such as *N-acetyllactosamine, acetylglucosamine, fucose, mannose* and *neuraminic acid* [16]. The primary structure of Lf glycans in polymorphonuclear cells (neutrophils) is identical to the glycans derived from human milk. **The high resistance of Lf to proteases and pH variations is partly attributed to glycosylation of the protein** [26]. This resistance is important during the transport of Lf from human milk through the intestine of newborns, which facilitates absorption of Lf from the colostrum [4,15,27].

Glycosylation sites are important to the antiviral capability of Lf because cell-surface glycans are frequently targeted by viruses to gain entry into human cells. Therefore, one of the protective effects of Lf released from neutrophils (as part of the innate immune response) is that it neutralizes the glycan-binding properties of certain viruses to prevent the entry of viruses into its target cells. This mechanism has also been hypothesized for bacterial toxins, which target human glycans.

Therefore, Lf released from neutrophils sequesters Fe and neutralizes the adhesive properties and the binding of viruses and bacterial toxins to glycan sites present in the host cells, which reinforces the role of Lf in the innate immune response [18,19]. However, further studies of these effects are needed.

5. Genes of Lactoferrin and polymorphism

Lactoferrin belongs to the Transferrin (Tf) family, whose genes have constitutive and inducible expression [28,29]. The Lf gene is located on chromosome 3p21.3 in humans, on chromosome 22 in cattle and on chromosome 9 in mice. The size of the gene ranges from 23 to 35 kb and contains 17 exons, 15 of which encode an amino acid sequence that is identical among different species [29]. The main differences are present in the mouse and bovine Lf genes, the resultant proteins differ by approximately 22 amino acids. Furthermore, bovine and porcine Lf have 1 or 2 amino acids fewer, respectively, than the human Lf [28,29].

The termination codon for intron-exon splicing is highly conserved in 12 of the 16 exons, and it is identical among human, mouse, bovine and porcine Lf genes [28]. **Human Lf begins with a glycine**

followed by 4 arginines, which makes it unique. This region is important for Lf's interaction with bacterial components and its bactericidal effect [28]. The Lf gene promoters in different species contain a TATA box with an adjacent SP1 site and the adjacent element GATAAA AP1/CEBP [28, 29**]. These promoters have multiple response elements to steroid hormones, indicating that the synthesis of Lf is differentially regulated by these hormones** [28].

Until now, genetic polymorphisms have been reported in the regulatory region of bovine and human Lf genes [28], although their importance and functional effects are still under investigation. Furthermore, polymorphisms within the coding region from the Lf gene have been described [28]. **Mutations in these regions can affect the protein structure and, in turn, alter its biological function.** In addition, polymorphisms that cause amino acid substitutions in the surface of the protein may not affect its protein structure but instead alter their surface properties [28]. **Alterations in the N-terminus of Lf are particularly important with regard to how they affect its biological activity** [28].

Lf structure exhibits many features in common with serum Tf, including the ability to bind iron with high affinity and a highly conserved 3D structure with sites for identical iron-binding [18]. In the iron release mechanism, the "trigger di-lysine," which is 2 lysines in Tf (Lys 206 and Lys 296) that are joined by hydrogen bonds in the N lobe, has the capacity to release iron more easily than human Lf, in which Arg 210 and Lys 301 are equivalent to the lysines mentioned above. However, these residues do not interact because Lys 301 adopts a different conformation by binding to Glu 216 to form a salt bridge, thus increasing the affinity and retention of iron [30].

Through bioinformatic analysis of the Lf genes from 11 species, changes in the amino acid residues of the protein were observed: 8 in Homo sapiens, 6 in *Mus musculus*, 6 in *Capra hircus*, 10 in *Bos taurus* and 20 in *Sus scrofa*, which had the most polymorphisms [31]. **This finding reflects the functional diversity of Lf derived from different species. The polymorphisms in Lf genes from humans and other species have some clinical implications.** In humans, the Glu-561-Asp polymorphism is associated with susceptibility to infections, such as **Herpes Simplex keratitis,** where the protective effect is provided by the Asp 561 variant [25 32]. Additionally, the polymorphism rs 1126478 AG genotype has been associated with localized **juvenile periodontitis,** in which the heterozygous carrier is associated with a more aggressive form of the disease. The onset of A reduces this aggression [33].

Similarly, this polymorphism has been associated with lower triglyceride levels and higher HDL cholesterol intake in men [34]. In addition, the rs 1126477 polymorphism has been associated with lower triglyceride levels in men when compared with the heterozygous AG G homozygotes [34]. **There are SNPs in genes Lf and IL-8 that are associated with the susceptibility of travelers in developing countries to "traveler's diarrhea"** [35].

Studies in **Irish cattle** have found differences in polymorphisms in the promoter region of GATA-1 from Sp1 transcription factors with respect to other populations [36]. In the **Holstein-Friesian** animal

population, the T/A SNP, which results in the replacement of Val89Asp in the mature Lf, has been identified [28]. Other SNPs were amino acid substitutions in the sequence of the peptide Lfcin B as well as an A/G SNP associated with an 181Cys Tyr change in the **Jersey** population [28]. **These polymorphisms may affect Lf gene expression levels and could be the key to mastitis resistance studies in these species** [28,37,39].

6. Lactoferrin-derived peptides and recombinant lactoferrins

Undoubtedly, the identification of peptides or amino acid residues responsible for the microbicidal action of Lf, which are generated naturally during its passage through the gut, was one of the most important discoveries in the field of research about Lf. This discovery occurred in 1982, when Wayne Bellamy and colleagues, from the laboratory of Nutritional Sciences, Morinaga Milk Co. of Japan, reported a fragment of Lf that was produced *in vivo* by the degradation of bovine Lf in the gut with the gastric enzyme **pepsin.** Later, studies showed that this fragment was responsible for the bactericidal action of Lf [39,40].

This group of researchers obtained the peptide in the laboratory from human and bovine Lf. **After digestion of Lf with pepsin or trypsin enzymes, the generated peptide was similar in sequence to one that was isolated and identified from human feces, indicating that it was produced *in vivo* as well *in vitro*.** Subsequently, they analyzed the sequence and found that the peptides consisted of 18 amino acid residues formed by a disulfide bridge of cysteine residues 20-37 from human Lf and residues 19-36 from bovine Lf. **This peptide was located in the amino-terminal domain or N1** [39,40].

The bactericidal activity of these peptides was tested in Gram-positive and Gram-negative bacteria. The peptides were more potent and effective than the native Lf, and they were bactericidal at lower concentrations used for the whole molecule. The bacteria that were susceptible to Lf and its derived peptides included ***Escherichia coli, Salmonella enteritidis, Klebsiella pneumoniae, Proteus vulgaris, Yersinia enterocolitica, Pseudomonas aeruginosa, Campylobacter jejuni, Staphylococcus aureus, Corynebacterium diphtheriae, Streptococcus mutans, Listeria monocytogenes*** **and** ***Clostridium perfringens*** [40].

Other important findings reported the effect of cations in the media which diminished the bactericidal ability of peptides. The peptide generated from bovine Lf was more effective because it diminished the growth of most of the tested bacteria. In contrast, ***Pseudomonas fluorescens, Enterococcus faecalis and Bifidobacterium bifidum*** **were not affected when they were incubated with Lf of Lf-derived peptides, suggesting that they were only specific for pathogens and not for commensals** [40].

These peptides were called Lactoferricins (Lfcins): Lfcin B, for the peptide derived from bovine Lf, and Lfcin H, for the peptide generated from human Lf. The active part of the Lf protein is present in these fragments in the amino terminus of the molecule. Other studies have reported other Lf

fragments located near the amino acids of these original peptides, but there are differences in some of the amino acids residues.

Bolscher and colleagues (2005) reported that a region of the amino acids located in the amino terminus, near the region known as Lfcin B in Lf, also has potent bactericidal activity in ***Bacillus subtilis, Escherichia coli,* and *Pseudomonas aeruginosa* compared with the native peptide** [41,42]. This peptide is called Lactoferrampin (LFampin) and consists of the amino acid residues 265-284 of bovine Lf [41]. **Lfampin also affected pathogen membranes, as demonstrated in *Candida albicans*.**

During 2007, Bolscher and colleagues fused LFcin17-30 and LFampin265-284 with a lysine to obtain a new peptide that was larger and more cationic than its constitutive peptides. This peptide was called lactoferrin chimera (LF chimera). The interaction of this peptide with a membrane model is stronger than the native Lf and constitutive peptides. Remarkably, this peptide is bactericidal at high salt concentrations. Cationic peptides typically lose their bactericidal activity in high salt concentrations, but this is not the case for LF chimera.

Our group has demonstrated that the **LF chimera was bactericidal in pathogenic species from Vibrio,** halophilic bacteria that grow in high salt concentrations [43]. Therefore, we concluded that this peptide was more effective than its constitutive peptides and native Lf; however, more experiments are necessary to explore its mechanism of action.

LF chimera was microbicidal in ***Escherichia coli, Streptococcus mutans, C. albicans* and *E. histolytica***. *In vivo*, lactoferrin chimera had protective effects in a mouse model of enterohaemorrhagic *Escherichia coli* O157:H7 infection [44]. **Because this peptide contains a higher net cationic charge, it most likely interacts with the negative charges in the membrane of microbial components.** This peptide adopts a three-dimensional structure similar to that of native Lf, which could confer advantages.

In light of the tremendous potential of Lf in medical treatments, some expression systems have been used for their large-scale production. In 1992, Ward and colleagues reported the **production of recombinant human lactoferrin (rhLf) in *Aspergillus oryzae*** [45]. Subsequently, a recombinant murine Lf was produced to study the species specificity of Lf activity in a mouse model. Van Berkel and colleagues (2002) reported the large-scale production of **recombinant human lactoferrin in the milk of transgenic cows** [46].

In the same year, a recombinant porcine Lf was obtained by Wang and colleagues, who used the ***Pichia pastoris*** expression model [47]. In addition, a recombinant **Lf expressed in rice** (rhLf), biologically active and similar to native human Lf was generated. The purified protein is indistinguishable from natural human Lf in terms of its three-dimensional structure and biological activities, including iron- and receptor-binding functions and anti-bacterial activity [48]. On the other hand, **genetic constructs containing the human Lf gene were created by Russian and Belarus scientists. By using these constructs, they generated transgenic animals that produce high hLf concentration in their milk** [49].

7. Lactoferrin receptors in human cells

Studies have described that the membrane receptors for Lf **(LfRs)** show **high affinity and specificity**. Furthermore, **LFRs do not interact with other proteins from the Transferrin´s family,** such as Transferrin, Ovotransferrin, or Ferritin. **Unlike TfRs, LfRs recognize the saturated and unsaturated forms of Lf,** although their tissue distribution is more restricted than transferrin receptors (TfRs).

Lf binds to certain neoplastic lines, mainly to human leukemia, erythroid and granulo-monocytic cell lines. Cells bind Lf regardless of their state of saturation with Fe, as observed in later studies. **In the promyelocytic cell line HL-60, mitogen stimulated differentiation into monocytes-macrophages was found that doubled up their ability to bind Lf with high affinity**. Additional studies determined that the LfR has a Mr of 120 kDa in the human erythroleukemia cell line K-562 [50]. **Cell lines bind holoLf to use as a source of Fe for growth.** However, the cells also recognize ApoLf at similar levels than the iron-saturated form. Therefore, more studies are needed to determine the effects from the interaction between Lf and its receptor in both physiological states of this protein [51].

In addition, there are LfRs in human lymphocytes because the cellular components from the adaptive immune system induce biological activities in Lf [52]. Receptor synthesis occurs after mitogenic activation with **phytohemagglutinin (PHA).** This activity was described for holoLf, which supported the proliferation of PHA-activated T Lymphocytes that were grown in iron-free conditions (without serum). **Interestingly, the binding of Lf to T cells was not affected by the degree of saturation with iron.**

Similarly, TCR γδ and αβ T cells, B cells and NK cells express LfRs after stimulation with polyclonal activators [53]. Preliminary characterization of the LfR using T lymphocytes indicated that it consists of two 100- and 110-kDa proteins. Studies have demonstrated **Lf endocytosis by T cells,** which stimulated T-cell differentiation from immature precursors. Lf also promoted the polarization of the Th1 or Th2 response according to whether tumor or infectious process was involved [52,54,55].

The first mammalian LfRs were described in macrophages. Initial studies demonstrated that holoLf binds to membrane components of rat macrophages through a receptor-mediated mechanism. **HoloLf released iron atoms into the cytosol of the phagocyte, which were stored by Ferritin.** After that, reports revealed that monocytes-macrophages contained LfRs, which reduce the free Fe^{2+} concentration during hypoferremia in infectious and inflammatory processes. However, **when Lf binds to surface nucleolin, it translocates to the nucleus and acts as a positive regulator of the synthesis of pro-inflammatory cytokines (IFN-g, IL-1b, IL-6 y TNF-a), which are required for the resolution of the infectious processes** [51,56].

In the small intestine, a specific LfR in the brush-border epithelial cells has been found [57,58,59]. This receptor has been observed in several mammalian species, such as rabbits, pigs, rhesus monkeys, mice and rats, and similarly binds the saturated and unsaturated forms with iron. Moreover, the optimal binding of Lf occurs at pH 5.5-6.0. **Divalent cations, particularly Ca^{2+}, are required for optimal binding to the brush border membranes.** The LfR is highly glycosylated and consists of a 130-kDa polypeptide chain with a pI of 5.8-6.0 [60].

The appropriate functioning of LfRs in the small intestine requires the intact distribution of Lf to epithelial cells in the gastrointestinal tract. **In humans, the digestive tract of infants has a relatively high pH and secretes low levels of enzyme, which allows for the innocuous transit of bovine and human Lf into epithelial cells** [56,57]. In contrast, **only 60% of bovine Lf resists proteolytic digestion mediated by pepsin, whereas human Lf is completely degraded in adults. However, neutrophil Lf and Lfs derived from pancreatic juices are discharged into exocrine secretions of mucosal surfaces.** Thus, **LfRs expressed in digestive-tract cells interact with Lf,** which shows diverse biological activities, including the **facilitation of Fe absorption, the modulation of mucosal immunity and the stimulation of mucosal differentiation** [60,61].

LfRs have also been identified in SV-40 epithelial cell lines derived from nonmalignant human breast tissue, benign breast disease cells, *hst* oncogene-transformed HBL-100 cells and breast carcinoma cells [62]. **The degree of binding affinity was reported to be similar in normal and tumor cells.** Importantly, the binding level was similar to activated T Lymphocytes, and the Lf receptor from mammary gland epithelial cells has common antigenic determinants with the receptor of activated lymphocytes. Subsequently, **the hormones that regulate the biosynthesis of Lf may influence the expression of LfRs in normal and malignant mammary epithelial cells** because the amount of mRNA of Lf is inversely proportional to the estrogen receptor [56,60].

In liver, hepatocytes bind Lf through a 45-kDa membrane protein (Lfbp). In non-activated platelets, saturable and reversible binding of Lf has been described [63]. The human platelet LfR was purified and presented similar physiochemical and immunological properties to the 105-kDa receptor of activated T lymphocytes. Data have shown the **presence of LfRs in the central nervous system;** however, further research is required to assess the possible involvement of Lf in the pathogenesis or resolution of neurodegenerative disorders because it usually accumulates in amyloid deposits [60,64].

The presence of Lfbps indicate Lf's multifunctionality in the body. Figure 1 lists the synthesis sites and forms of Lf, as well as its isoforms and LfRs found in the human organism.

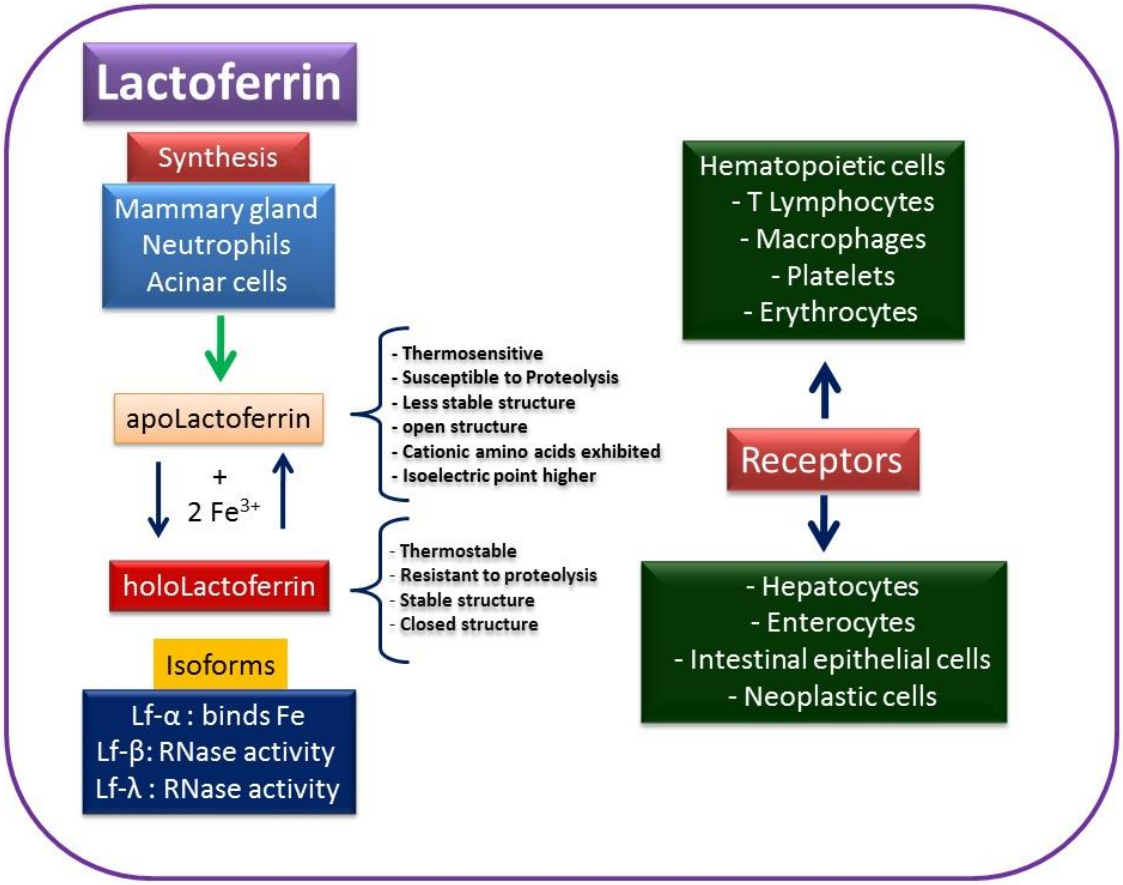

Figure 1. LACTOFERRIN IS SYNTHESIZED IN MAMMARY GLANDS, ACINAR CELLS AND THE SECONDARY GRANULES OF NEUTROPHILS.

Lactoferrin is released in its iron-free (ApoLf) form and has a high affinity for iron. When ApoLf binds iron (holoLf), the protein undergoes a drastic conformational change, which may alter its functions. All synthesis sites contain the three Lf isoforms, but only the alpha isoform has iron-binding capacity. The human body has many cells that contain Lf receptors.

8. Role of Lactoferrin in the human immune system

Lactoferrin is one of the components of the innate immune system, and *in vivo* and *in vitro* studies have clearly shown the regulation of the immune response and protective effects mediated by Lf against infections and septic shock [65]. Little is known about Lf's mechanisms of action as an immunomodulator. At the cellular level, **Lf modulates the migration, maturation and function of immune cells** [66].

At the molecular level, in addition to its ability to bind iron, Lf controls oxidative stress produced by cells in the immune response against pathogens and transformed cells. Lf interacts with several soluble components and molecules in cell membranes, which are responsible for its activity as an

immunomodulator [27, 67]. Lf also interacts with macromolecules and proteins such DNA, albumin, lysozyme, and IgA.

Due to its high isoelectric point, **Lf can be recognized by pseudoreceptors and thus participate in signaling events to induce, inhibit and promote the functions of cells in the immune response.** Table 1 lists some of immune functions that Lf regulates.

9. Lactoferrin and iron metabolism

The **roles of Lf in iron absorption in the intestine, especially in newborns, have been previously described and include the following: increased iron bioavailability and a high concentration of Lf in human milk** [3]. Nevertheless, some groups that study Lf consider these data to be controversial.

The presence of a specific binding site for human Lf in the microvilli of enterocytes, which may have characteristics similar to those of receptors, has been described [76,77,78]. However, studies have shown that there is no positive effect of Lf on iron absorption in the intestine and that Lf may even inhibit iron uptake [79,80]. The **removal of Lf from milk increases the bioavailability of iron** [81]**; therefore, it has been proposed that Lf plays a role in the control of iron absorption** [82]. Thus, **in the newborn gut, which has a great ability to absorb iron, Lf prevents excessive absorption.** Later, when the bowel develops and the iron needs of the child increase, Lf releases the bound iron after it is degraded by digestive enzymes [82].

In the last few years, research has demonstrated that **human Lf can bind ceruloplasmin, the blood serum ferroxidase,** *in vitro* and *in vivo* [83, 84]. This interaction produces an increase in the ferroxidase activity of ceruloplasmin [84]. **Lf and ceruloplasmin are acute-phase proteins and protect tissues from damage from free radicals found at the site of inflammation** [84]. Furthermore, **the affinity between these two proteins facilitates iron acquisition by apoLf at the inflammation site, thus preventing iron uptake by microorganisms** [83].

Table 1. DEFENSE AND IMMUNOMODULATORY FUNCTIONS OF LACTOFERRIN.

Function	Proposed mechanism	Ref.
Stimulation of adhesion and accumulation of neutrophils at damaged sites	Reduces the surface charge and repulsive forces in these cells	[68]
Modulation of migration, maturation and function of antigen- presenting cells	Unknown, it is postulated to be cytokine release	[54,55]
Promoting cell-cell interaction in granulocytes	Lf binds to the surface of these cells and reduces their charges	[69]
Increases or decreases the production of free radicals		[3,70]
a) In acidic environments, as in the phagolysosome, Lf promotes the formation of free radicals	a) Provides Fe	
b) In extracellular neutral environments, it decreases free radical production and thereby prevents oxidative damage	b) Lf sequesters Fe	
Through regulatory mechanisms, affects host defense mechanisms		[3,65,68,71]
a) Inhibitory effects - Inhibition of lymphocyte proliferation induced by mitogens and alloantigens - Blocks histamine release - Inhibits antibody synthesis - Controls the monocyte-macrophage activity - Anti-complement action - Increases natural killer cell-mediated cytotoxicity and lymphokine-activated killer cells cytotoxicity	a) Unknown mechanism, apparently depends on the activity of Lf as a supplier and chelator of iron	
b) Affects the secretion of cytokines - Inhibits the secretion of granulocyte-monocyte colony-stimulating factor - Inhibits the secretion of cytokines such as TNF-a, IL-1b and IL-2 in a concentration-dependent manner by partially saturated Lf - Affects the release but not the biological function of cytokines - In the presence of LPS, increases the production of IL-1b, TNF-a, IL-6 and prostaglandins	b) Unknown, depends on the saturation or release of iron	
- Can modify the inflammatory response in systemic lupus erythematosus through DNA binding	Lf and DNA interaction, prevention of anti-DNA-binding. Lf causes dispersion of antiDNA-DNA binding	[72]
- Increases the T-cell autoreactivity associated with arthritis	Cross-reactivity between Lf and the 65-kDa mycobacterial heat shock	[73]

induced by mycobacteria	protein	
- After being released from macrophages, increases phagocytic and microbicidal capacity in these cells	Macrophages ingest polymorphonuclear cells rich in Lactoferrin and increase their microbicidal capacity	[13, 27]
- Promotes motility and ability to form superoxides in polymorphonuclear cells	Unknown, it is proposed the participation of Lactoferrin with or without iron, and is prevented by anti-Lf	[55]
- Potentiates the microbicidal activity of peptides bactenecins, arginine-rich peptides located in neutrophil granules	Synergism between Lf and bactenecins, permeates bacterial membrane with increased efficiency	[13,74,75]
- Increases microbicide capacity of Lf and lysozyme		
- Activates the classical complement pathway	Unknown, it has been proposed that depends on adherence of Lf to membranes	[54,55]

Once iron is captured by Lf, this complex is internalized by macrophages, where iron is transferred to ferritin for storage, and then Lf is released again into the extracellular medium [85,86,87]. **However, the uptake of iron** bound to Lf by macrophages **is very low,** and this protein, after binding to monocytes, loses some of its ability to bind these cells again [85]. **Lf also plays a role in inflammation as a mediator of the adhesion of neutrophils to the endothelium** [88].

Human milk is especially rich in Lf; its concentration in colostrum is approximately 7 g/l, remains elevated throughout lactation at approximately 1 g/l, and increases at the end of lactation to values of 20-30 g/l [6]. Similarly, in bovine milk, the levels change throughout lactation, but the final levels are much lower, approximately 0.1 g/l [6].

Therefore, milk for infant feeding prepared from cow's milk is deprived of Lf, and in some countries such as Japan, Lf is added to infant formula. Currently, these milks are supplemented with bovine Lf obtained from **lactoserum** because human Lf is not widely available. Although the protective effect of lactoferrin obtained from bovine lactoserum (of different species and most likely submitted to different thermal treatments that are not controlled by the producer of infant formula) in children is not yet known, the use of human Lf would be preferred.

10. Synergism of lactoferrin with antibiotics and other components effectors of the immune system.

Lactoferrin acts synergistically against pathogens with other immune system components such as immunoglobulin A (IgA) and lysozyme [74,89]. The action may be explained in part as the **sum of two different mechanisms. First, the highly cationic nature of Lf confers iron-binding capacity and allows it to bind to cell surfaces and anionic molecules** [90,91,92]. Second, **its serine-protease, immunomodulatory and immunostimulatory activities, along with the other components' mechanisms of action,** could be responsible for the synergistic effect.

Because **Lf and peptides interact on the membrane of some pathogenic bacteria, they are more susceptible to the effects of lysozyme and IgA,** which act synergistically [75,93]. The effect of Lfcin has also been studied and has been shown to have the same function than Lf, but it is most effective [94,95]. Administration of iron to the nursing mother does not interfere with Lf saturation in milk. **The first days of lactation may also exert a protective role for the mammary gland itself.** Recent studies have shown that breastfed children have higher levels of IgA, which stimulates the immune response, than infants fed with artificial formula [96].

The majority of antimicrobial agents are toxic. Some may be mutagenic, and microorganisms may develop resistance to them. Antibiotics' mechanisms of action against bacteria include the inhibition of protein synthesis, DNA damage, modification of the cell wall, changes in membrane permeability or general structure, and enzyme inhibition. By combining the effects of Lf and the derived peptides, which directly affect membranes, **the antibiotic effect will occur faster as a result of these synergistic mechanisms** [74,97,98].

Furthermore, the peptides are potential immunomodulators and immunostimulators; hence, their combined use with antibiotic results in a very attractive therapy [99,100,101,102]. Figure 2 illustrates the different proposed functions for Lf.

11. Applications of lactoferrin and perspectives

Lactoferrin was described in the late 1930's as a bovine milk protein of unknown function and was isolated in the 1960's as a protein from the red fraction related to Tf. Later investigations described its functions and potential applications, which stimulated the study of this protein and led to its large-scale production. Figure 3 lists the important events in the study of Lf throughout the decades.

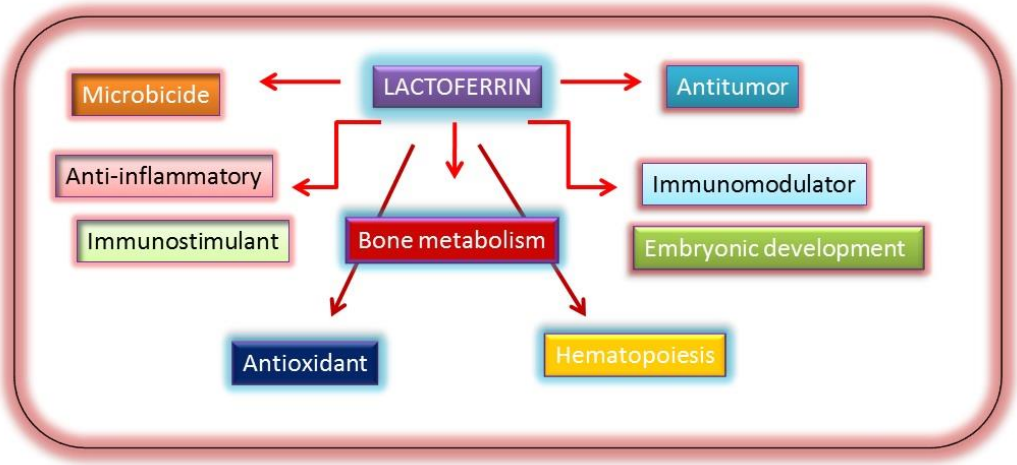

Figure 2. LACTOFERRIN AND ITS BIOLOGICAL FUNCTIONS.

Lf is microbicidal, acts as an immunomodulator and immunostimulator, has antitumor and antioxidant activity, and plays an important role in hematopoiesis, embryonic development, development and bone metabolism, which are the most well-studied functions.

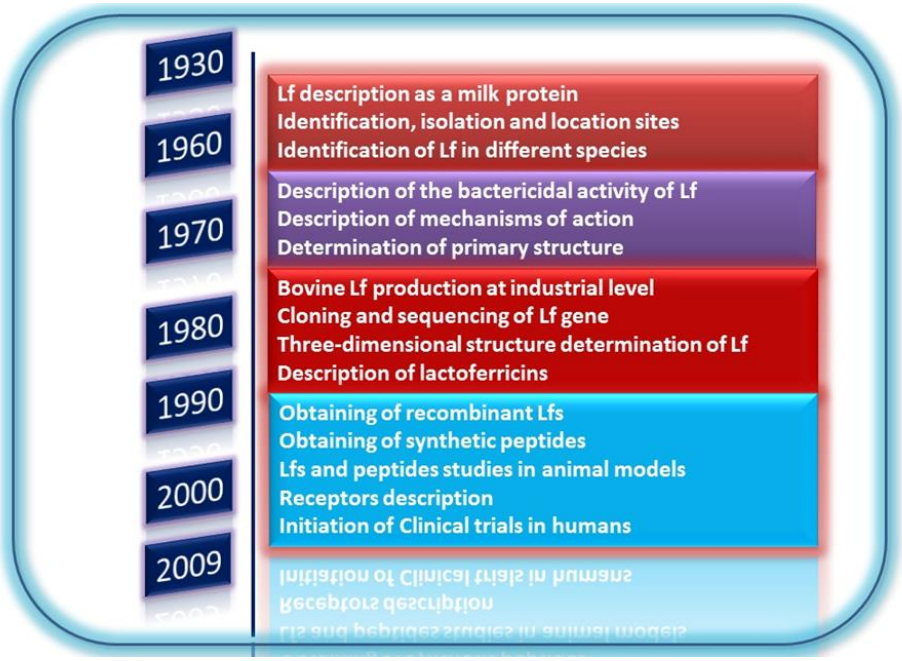

Figure 3. CHRONOLOGY OF KEY EVENTS IN THE RESEARCH FIELD OF LACTOFERRIN.

Currently, the results of basic science have led to clinical trials because the *in vitro* results correspond to those found in humans and other mammals. For example, mice were infected with strains of ***Clostridium*, enterobacteria, *Escherichia coli*, *Helicobacter*, *Candida*, Influenza virus and *Entamoeba histolytica* in *in vivo* studies** [103,104,105,106]. Animals treated with Lf **can resolve infection**; the authors argue that Lf both directly affects the pathogen and inhibits interaction with target cells.

In addition to direct microbicidal activity and inhibition of adhesion of pathogens to host epithelia, Lf stimulates, modulates and regulates the immune response in some animal models. In mice submitted to stress and treated with chemotherapy agents to destroy their cells, treatment with oral administration of Lf reconstitutes their humoral and cellular response in a short time [107]. In *Sthapylococcus aureus*-infected animals, treatment with Lf increased the Th1 response [108].

In biological models established in animals to investigate Lf's anti-inflammatory roles, Lf produced the expected response [109]. As a prophylactic, Lf reduces mortality in a neonatal rat model of co-infection with *Candida* and *Staphylococcus* [110]. In a model of **oral candidiasis,** Lf stimulates the immune response to resolve the infection.

In carcinoma and tumor models in animals, Lf exhibits a direct effect against cells [111, 112] and stimulates the immune response to destroy tumors. In animal models of damaged epithelium, Lf participates in tissue repair, such as cornea restoration, skin healing and bone and joint regeneration.

Clinical trials have been conducted in patients, both for treatment and prophylaxis of infections and carcinomas. Recently, the first preliminary assessment of a cream containing Lf to treat women with **vulvovaginitis caused by *Candida albicans*** was initiated [113]. In a pilot study with elderly patients, toothpaste that contains Lf in addition to other microbicidal peptides did not generate conclusive results, raising the possibility of a new study with a better design [114].

In patients with hepatitis C, interferon therapy combined with ribavirin and Lf decreased viremia and viral RNA titers compared with patients who did not receive therapy [115]. **In a study of patients receiving stem-cell transplants, who were susceptible to infections and inflammatory reactions, Lf had anti-inflammatory and anti-microbicidal effects** [116].

The effectiveness of Talactoferrin-*α*, a recombinant form of human Lactoferrin, has been evaluated in phase 1 and 2 clinical trials in diabetic patients with neuropathic ulcers. The results showed that patients improved without any adverse effects after the protein was topically applied to the patients' ulcers [117]. **The same protein was used in a cohort of patients with metastatic kidney cancer. Talactoferrin was well tolerated and showed no toxic effects, and the patients had a longer and better quality of life.** The authors propose that given the results of this study and other preclinical studies that demonstrate the effectiveness of talactoferrin, a randomized, double blind, multicentered and controlled clinical trial must be conducted [117,118,119, 120].

Similar results were found when talactoferrin was used in patients with head and neck cancer; in addition, **bovine Lf has been evaluated in patients with colon cancer and showed chemoprotective and chemopreventative effects** [121]. **Other clinical trials are performed using recombinant Lf to treat diarrhea in children, to prevent infections in neonatology services in pediatric hospitals, and as an adjuvant in patients with cancer, diabetes and viral infections such hepatitis B and C.**

The use of Lf as either as prophylactic or therapeutic should have promising results in clinical trials, whether as a **monotherapy** or in combination with current therapy. **In a systematic review that evaluated the clinical trials in which Lactoferrin was used, the authors could not strongly conclude whether Lf works or not. However, as more research and clinical trials with better designs are performed, Lf may be used as a therapeutic or prophylactic for certain diseases.**

12. Conclusions

Although much remains to be learned about Lactoferrin and its derived peptides, Lf is one of the proteins of the innate immune response that has better prospects for clinical application as a prophylactic and therapeutic of infectious and inflammatory diseases and certain types of cancer and as a stimulator of hematopoiesis and the immune system. However, microorganisms can grow if Lf is used with iron, but ApoLf has the most beneficial functions attributed to this protein. As a natural protein, Lf is well tolerated and does not cause complications; additionally because it can be obtained synthetically or by recombinant techniques, the use of Lf as a food additive in infant formula and other health products will increase.

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Chapter 8

Ferritin and the safekeeping of iron in the cell

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Abstract

Iron is a vital element for most cells, because it participates in all cellular redox processes. This element cannot be free in the cytosol, because it originates reactive oxygen species. Most organisms possess an iron regulation system, where Ferritin plays the role of storing this element.

Ferritin is a cytosolic protein capable of storing up to 4500 iron atoms. This iron rich protein can be used as an important source of iron not only by several cell types which have an imperative need for this element, but also by pathogens.

There have been only a few studies on Ferritin endocytosis and its binding to other proteins or receptors. These studies have been made in human cells only: hepatocytes, oligodendrocytes, B and T lymphocytes, and Caco-2 cells. These cells use Ferritin as an iron source and, via a Ferritin binding protein, internalize it through an endocytic process mediated by clathrin-covered vesicles.

Key words: endocytosis, ferritin, Ferritin receptors, iron, iron release, iron storage.

1. Introduction

Throughout evolution, virtually all cells have needed iron (Fe) to undergo the biochemical processes essential for life. This is because iron participates bound to proteins in a multitude of metabolic processes. One case is the enzyme **Ribonucleotide reductase,** required for DNA synthesis. Another example is the protein **Hemoglobin,** in charge of transporting oxygen from the pulmonary alveoli to all cells through blood. In this protein, iron is an integral part of the molecule.

In spite of its essential role in cells, **iron may have a toxic effect.** When it is released from the protein, it produces reactive oxygen species (**ROS**) through the **Fenton reaction. One of** ROS is **hydroxyl radical** (**OH·**), which is able to damage a large quantity of cellular components, including DNA, membrane lipids, and proteins. This toxicity is the reason by which all organisms have evolved systems to chelate, transport and store iron. In mammals, three main proteins participate in these processes: **Lactoferrin, Transferrin and Ferritin**.

Lactoferrin is localized in mucosae. This protein is responsible for chelating free iron, thus not only avoiding its toxicity to the cell, but also sequestering it from pathogenic microorganisms to prevent they use this element to cause infection. **Transferrin is located in serum and lymph**. This ferric protein transports iron from enterocytes, the main absorption site of iron, to all cells that need it. **Ferritin is found in cytosol. This protein is the main cellular iron storage and detoxifies the cell by preventing iron to be free.**

In this chapter we will talk about Ferritin, its general properties, synthesis and regulation. Also about iron storage and release by this protein, Ferritin binding proteins in several cellular types, Ferritin internalization pathways in eukaryotic cells and the receptors involved in this endocytic process.

2. Ferritin structure and function

Ferritin main function is to store iron used for cell growth. Furthermore, when intracellular iron concentration increases, **Ferritin contributes to detoxify the cell by sequestering iron inside its cavity,** thus preventing the element to be free in the cytosol. Additionally, **Ferritin is capable of storing up to 4500 Fe atoms per molecule** [1,2] and has the ability to control the reversibility of the transition phase between soluble ferrous iron and ferric iron mineralized inside its cavity [3,4].

Ferritin is a versatile protein, mainly cytosolic but also localized in other sites such as mitochondria, nucleus and serum in higher eukaryotes. Interestingly, Ferritin is found in all three domains or super-kingdoms in which living beings are classified: **Archaea, Bacteria and Eukarya**. In the latter, it is present only in some fungi but not in yeasts [4,5].

Apo-Ferritin (without iron) is a heteropolymer with an average Mr of 450 kDa. It is composed of 24 subunits of two types, the light and the heavy subunit [6,7]. The heavy subunit (H) has a Mr of 21 kDa (178 amino acids) with **Ferroxidase** function and an intrinsic **Ferrireductase** activity [8]. This is the initial binding site of iron through electrostatic interactions [9]. The light subunit (L) has a Mr of 19 kDa (174 amino acids) and its function is to form the iron core, whose interior possesses a negative charge conferred by 9 glutamic acids [10] (**Figures 1 and 2**).

Ferritin is a glycoprotein with great heterogeneity in carbohydrates. These are present in mainly two types: one contains fucose, mannose and galactose and the other glucose exclusively. It is important to note that only plasma proteins contain mannose, and very few proteins include glucose in their structure. **One of the functions ascribed to Ferritin's carbohydrates is their role in its own secretion, as it happens with insect and human serum Ferritins, which are mainly formed by the L subunit** [11]. Ferritin´s secondary structure is 50% α-helix, 37% β-sheet and 11% random structure [6,7,8]. Secondary structure of both subunits is very similar, although they are only 55% identical [4].

As previously mentioned, Ferritin has remained **highly conserved during evolution. I**t is present in eukaryotes, e.g. animals, plants and insects, and also in prokaryotes. There are however some structural and functional differences, since they share only 15% of sequence identity [5]. **One of the most important differences is the regulatory mechanism in vertebrates.** This regulation is at the level of transcribed mRNA, in sites known as **"iron responsive elements"** (**IREs**), found in 5' and 3' untranslated regions (**UTR**). Ferritin genes present in plants, yeast and bacteria, do not have IREs [4,8].

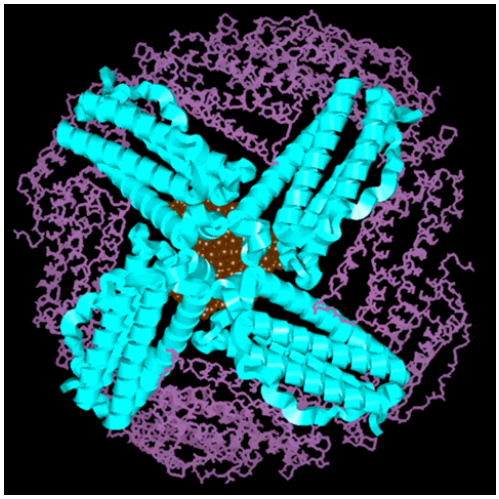

Figure 1. FERRITIN MOLECULE Taken from http://www.chemistry.wustl.edu

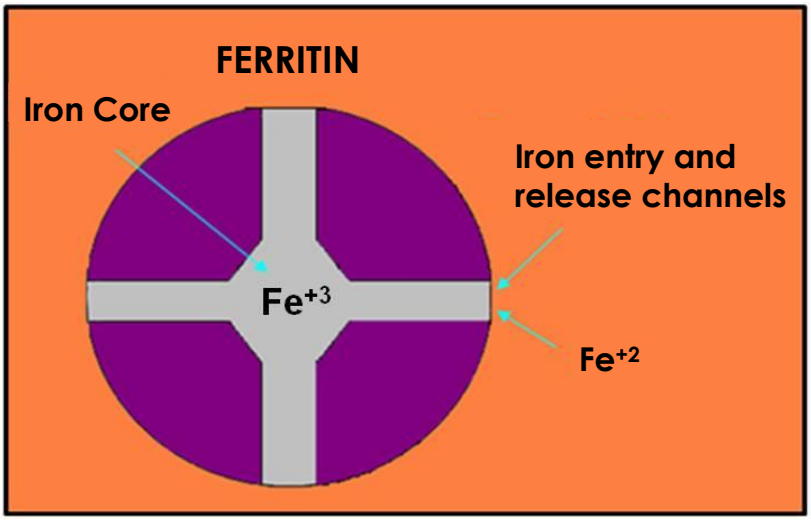

Figure 2. SCHEME OF FERRITIN MOLECULE WITH ITS IRON CORE

3. Iron storage by Ferritin

Iron storage by Ferritin is well characterized. This process is carried out in three consecutive stages:

Binding of Fe^{2+} to the H subunit

Oxidation of Fe^{2+} to Fe^{3+}

Formation of the iron core inside Ferritin [10,12]

The first stage or **Fe^{2+} binding to the H subunit** is carried out through **electrostatic interactions between iron and the glutamic acids** in the H subunit. This step is very fast as it happens in milliseconds.

The second stage or **oxidation of Fe^{2+} to Fe^{3+}** is carried out by the H subunit, which features a ferroxidase enzymatic activity. This phase is slower than the first; **it happens in seconds**.

The third stage of the process is the **formation of the iron core**, which is accomplished with the help of the two subunits, since a maximum incorporation of iron requires 18–30% of the H subunit. Another important part at the third stage is the association of the subunits, where they form **anti-parallel rhomb-shaped pairs**. Iron is stored bound to negative amino acid residues in the inner surface of the L subunit, thus forming the iron core. This is the slowest stage of the iron storage process by this protein, since it **is performed in minutes** [4,10,12,13].

Recent studies have found a certain **chaperone protein**, which **facilitates binding and storage of iron in Ferritin, *in vivo* and *in vitro***. This chaperone was identified as a binding protein to poly-(rC)-1

(**PCBP1**), which is an RNA binding protein expressed ubiquitously in mammalian cells. It is found in the cytosol and nucleus [14].

4. IsoFerritins

Cells have different iron needs, and thus Ferritin has adapted by evolving several **isoforms**. These different forms of Ferritin are originated by **different proportions in the number of H and L subunits** [4, 5].

The most efficient Ferritins for *in vitro* iron incorporation are those found in the tissues of **liver and spleen.** These Ferritins contain 80-95% of the L subunit and 5-20% of the H subunit. Other tissues with lower iron storage needs contain a large percentage of H subunit. Therefore they possess a greater ability to detoxify the cell under iron excess conditions. The proportions of H and L subunits in a specific cellular type do not remain constant. Moreover, they can change during certain processes such as differentiation or several pathological states [4, 5].

5. Regulation of Ferritin expression

The need to store iron and reduce oxidative stress is the stimulus for regulating Ferritin synthesis. Regulation in mammals is both at **transcriptional and post-transcriptional** level, whereas in plants and bacteria it is only at the **transcriptional** level. A scheme is shown in **Figure 3**.

5.1 Transcriptional regulation of Ferritin

Currently, there are few studies on the transcriptional regulation of Ferritin. Oxidizing agents in the cell generate a large induction of Ferritin transcription, especially the H subunit. **Transcription factors** activated by oxidative stress: **Nrf2, JunD** and the **co-activator p300CBP bind to antioxidant-responsive elements (ARE) located in the 5' end of Ferritin genes** [17].

Hintze *et al*. demonstrated that the **transcriptional regulators Bach1** and **Maf** bind to AREs and repress Ferritin synthesis. These regulators are inactivated by the Heme group and Ferritin repression is reverted [17]. **Cytokines TNFα** (tumor necrosis factorα) **and IL-1α** (interleukin-1α), **induce Ferritin H transcription through nuclear factor NFκB. In contrast, c-myc decreases Ferritin H transcription** [19].

5.1 Post-translational regulation of Ferritin

Ferritin synthesis process occurs in a concerted way with the **Transferrin receptor (TfR)** synthesis in different cellular types, *i.e.* **when Ferritin synthesis is activated, TfR receptor synthesis is repressed.**

Another factor influencing Ferritin synthesis is intracellular iron concentration, where iron regulatory proteins **IRP-1** and **IRP-2** play a crucial role in regulation [37]. IRP-1 and IRP-2 **control Ferritin and TfR synthesis, by binding to IREs located in 5' and 3' UTRs of their mRNAs**. IRPs bind to these consensus sequences in Ferritin and TfR mRNAs with the same affinity [4,38,39]. **When intracellular iron concentration decreases, the IRPs bind to IREs repressing Ferritin mRNA translation,** and conversely, **stabilizing TfR mRNA, thus increasing its half-life and therefore its level of translation** [40].

When intracellular iron level is high, there is a decrease in the binding capacity of IRPs to IREs in the mRNA. **IRP-1 and IRP-2 are regulated differently.** When intracellular iron levels rise, IRP-2 is rapidly degraded [41,42], but IRP-1 diminishes its binding capacity to mRNA and detaches from it at the same time.

IRP-1 has a Fe-S group that senses the intracellular iron concentration. When it is low, IRP-1 loses the iron atom in the Fe-S group causing the binding to IREs. This phenomenon results in the repression of Ferritin synthesis and stabilization of the RTf transcript. On the other hand, when intracellular iron levels rise, the IRP-1 recovers the iron atom from its Fe-S group causing the opposite effect [43,44,45].

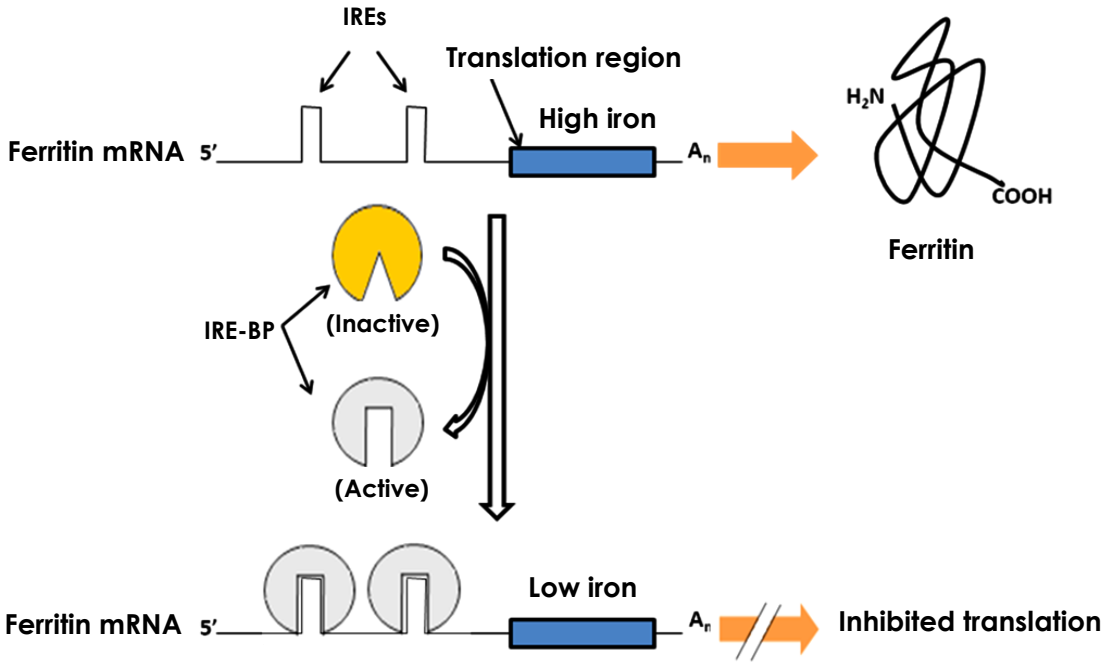

Figure 3. REGULATION OF FERRITIN SYNTHESIS

6. Ferritin iron release

Currently, two mechanisms have been proposed for Ferritin iron release. This element can be **released from the intact Ferritin** or **released after protein degradation**. The relative importance of each mechanism is unknown, although several studies indicate that it is mainly through Ferritin degradation [5].

Yang *et al*. conducted *in vitro* studies, whose results suggest that the first step of iron release is its **reduction.** Therefore, iron is transformed to the Fe^{2+} soluble form, which must be rapidly trapped by a chelating agent to be transported out of the protein and finally released [13,15]. Other studies describe that the H subunit has an intrinsic **Reductase** enzymatic activity, suggesting that **the same H subunit oxidizes and reduces iron according to the cellular needs** [9]; also, it has been described that **the same iron entry channels serve for iron release** [4,16].

Over expression of **Ferroportin (Fpn)**, an exporter of iron to the cytosol, induces **Ferritin mono-ubiquitination**, resulting in its **degradation by the proteasome** [20,21]. Ferritin degradation in the cytosol results in iron release from Ferritin. This, in turn, gives to the cell´s reduction system (**$FMNH_2$**), a direct access to Fe^{3+} ions, resulting in a complete dissolution of the iron core [4].

The **hematopoietic and reticuloendothelial system cells** have high iron content. In these cells Ferritin is found in agglomerates that protect the protein from **cytosolic degradation**, but may also **stimulate the entry of Ferritin to lysosomes**. In this case, iron cannot be released easily, because it has been separated from the $FMNH_2$ reduction system. This will result in aggregations of Ferritin cores and the formation of **Hemosiderin,** which can prevent the uncontrolled release of iron [4]. It has also been observed that acute iron decrease by chelators, increases Ferritin degradation by the lysosome [22,23,24].

7. Nuclear Ferritin

In different cell types, **cytosolic Ferritins** rich in H subunits are present **transiently in the nucleus. T**his is caused by several factors, such as **abnormal iron increases, pro-inflammatory cytokines and oxidative stress** [5]. No nuclear localization signal has been found in Ferritins rich in the H subunit, but it is believed that a specific sequence in the H subunit is involved in this process, since only the H subunit rich Ferritins are translocated into the nucleus.

It is predicted that **the O-glycosylation is the mark for Ferritin translocation**, since all nuclear Ferritins are O-glycosylated [25]. In chicken corneal-epithelial cells, it was determined that the translocation mechanism is mediated by a protein called **Ferritoid**, which has a **Ferritin binding domain and a nuclear localization signal** [26].

Once inside the nucleus, H-subunit rich Ferritins can form a **stable complex with DNA**, without a specific binding sequence, thus **protecting DNA from oxidative damage** [27]. Alternatively, Ferritin can donate iron to the enzymatic activities in DNA and may play a role in **regulating the transcription of specific genes** [4].

8. Mitochondrial Ferritin

In most cellular types, the concentration of mitochondrial Ferritin is low, with the exception of testis and some specific physiological conditions [28,29]. Mitochondrial Ferritin is related in structure and function to the cytosolic one. Nevertheless, they are different in some aspects: **mitochondrial Ferritin is a homopolymer of a 30 kDa subunit, similar to cytosolic H subunit.** This subunit features a 60 amino acid peptide involved in the **translocation into mitochondria**, where this signal sequence is removed [29]. Ferroxidase activity is significantly lower than in cytosolic Ferritin, although the ferroxidase center is conserved. This is due to the fact that only 12 ferroxidase centers are active.

Mitochondrial Ferritin has high affinity for iron, and when it is over-expressed, it competes for this element causing a decrease in its cytosolic concentration. Additionally, this leads to an increase in the binding of IRPs to IREs, resulting in a **decrease of cytosolic Ferritin levels and an increase in TfR**, even though functional implications are not defined [30,31]. The stimulus to mitochondrial Ferritin expression remains unknown; although there is an increase in the synthesis of mitochondrial Ferritin in pathological cell types where mitochondrial iron accumulation is evident, like in the **ringed sideroblasts** [25, 32].

Mitochondria contain a large quantity of iron. Most of the metabolically active form of this element in the cell is processed there for the **synthesis of Heme and Fe-S groups**. In the mitochondria, a great amount of oxidative stress is originated by the oxidative phosphorylation and respiratory chain. Hence, mitochondrial **Ferritin provides protection to the mitochondria not only** under normal conditions, but also when iron homeostasis is lost [4].

9. Extracellular Ferritin

In addition to cytosolic Ferritin, there is Ferritin in plasma. It is thought that Ferritin is trafficked into circulation through secretion of Ferritin by cells or through the release of Ferritin from damaged cells. Plasma Ferritin concentration varies from 13-300 µg/l. The L subunit present in this Ferritin form is rich in glycosilation and contains hardly any or no iron at all. This hold true even for conditions where iron is found in excess [4].

The regulation and function of plasma Ferritin are unknown; however, under pathological conditions, there is a relationship between plasma Ferritin and the amount of stored iron. For example, **under conditions of iron overload there are elevated levels of plasma Ferritin.** Aside from this condition, other factors affecting levels of plasma Ferritin are: tissue necrosis, damage to Ferritin-rich tissue, inflammation, infection, neoplastic disease, oxidative stress and increased turnover of red blood cells [4]. **Plasma Ferritin has been implicated in regulation of inflammation** [33], **differentiation** [34,35] and **immunomodulation** [36], although the mechanism has not been described yet. Up to now, the specific origin of plasma Ferritin remains unknown.

Plasma Ferritin concentration is given by the ratio of its secretion and elimination. **Hepatocytes are responsible for elimination of this kind of Ferritin,** since this cell type has specific receptors for both glycosylated and non-glycosylated Ferritin [4].

10. Ferritin receptors

Interestingly, **Ferritin is an important source of iron for the body's own cells.** This has leaded some researchers to devote themselves to the study of potential **receptors to Ferritin (FtR)** in different cell types. Natural Ferritins and the **homopolymers of recombinant human H and L subunits (H-Ferritin and L-Ferritin)**, have been used to study the presence, specificity, and expression of Ferritin binding sites on the surface of cells [49].

In the eighties, the existence of FtRs in different cells was described. Studies in **guinea pig reticulocytes** by Dr. Bligth in 1983, demonstrated the existence of a Ferritin binding protein **(Ftbp)** with a specific **affinity constant (Ka)** of $1.7x10^{-7}$ M. These cells possess between 30,000-130,000 **Ferritin binding sites**. Ferritin is used as an iron source in these cells since Ferritin-iron is utilized in mitochondrial heme synthesis [46].

In 1988, Dr. Adams's group [47] described the existence of **a Ftbp in human liver**. This protein weighs 53 kDa and has a Ka for Ferritin of $6.0x10^{-8}$ M. The specificity for Ferritin was confirmed in **competition assays** with other proteins. In the same year, this group [48] reported in **pig liver** a Ftbp of 53 kDa, with a Ka of $2.9x10^{-9}$ M. Its specificity for Ferritin was also confirmed by competition assays.

Studies by Dr. Fargion in the **proerythroleukemia K562 human cell line** showed the existence of an H-Ftbp that has a Mr of 100 kDa, with a Ka of $3x10^{-8}$ M. The cell line was estimated to have from 17,000 to 20,000 binding sites per cell for H-Ferritin. This group argues that **this receptor expression is modulated by the proliferation and differentiation status of the cell** [49].

This same group found a binding protein for H-Ferritin in **human lymphocytes CD4+, CD8+ and B cells stimulated with phytohemagglutinin (FHA). T**hese types of cells have specific binding sites for H-

Ferritin. Furthermore, by **flow cytometry,** they found that **Ferritin binding sites** are present in cells expressing **proliferation markers**, such as HLA-DR, MLR3, IL-2 and TfR. Because picomolar concentrations of H-Ferritin inhibit **blastogenesis** induced by FHA, these researchers concluded that the **binding sites for Ferritin could serve as proliferation markers**, with the unusual function of down-regulating cellular proliferation [50].

In 1992, Dr. Moss described in a **human cell line of T lymphoid lineage (MOLT-4)**, the existence of a binding protein for H-Ferritin. This Ftbp has a Ka of $6.3\text{-}6.7 \times 10^{-7}$ M. This cell line has from 6,000 to 15,000 binding sites for H-Ferritin [51].

Recent studies in **erythroid lineage** precursor cells have described the existence of a **binding protein for H-Ferritin** with a Ka of 4.1×10^{-8} M. This binding protein **internalizes Ferritin**, which is used as an iron source by the cell. It was determined that internalization of Ferritin by these cells causes the inhibition of TfR expression, suggesting that the expression of the Ferritin binding protein and the TfR are closely coordinated by common regulatory elements. In this paper, it is concluded that **the internalization of Ferritin by these cells is an alternative route of iron intake**, **in addition to the classical pathway of Transferrin internalization** [52].

In 1999, Dr. Hulet demonstrated the existence of **binding sites for H-Ferritin** in both the **white matter of adult mouse brains** [53] and **in human brains** [54]. In mouse brains, the Ferritin binding sites have a Ka of 4.65×10^{-9} M. Moreover cells of the mouse white matter show a density of binding sites of 17.9 fmol bound/µg of protein [53]. In the case of the human brain, the Ftbp has a Ka of 3.5×10^{-10} M and a density of binding sites of 116.7 fmol bound/µg [54]. In both cases, **Ferritin functions as an important iron source**.

Recently, Dr. Chen described in **human cells of the liver, kidney and in B lymphocytes**, the existence of a receptor **TIM-2** (T cell immunoglobulin-domain and mucin-domain). This is a well-characterized **Tyrosine-kinase receptor**, which serves primarily as an **immunomodulator**, since it is involved in **airway hypersensitivity and in Th2 cytokines production.** This study found that the **receptor binds H-Ferritin** and **internalizes it through endosomes.** This is the first report in literature that describes a true FtR. Interestingly, these researchers were studying the receptor activation and not Ferritin as its ligand [55].

11. Ferritin endocytosis

Aside from the description of Ferritin receptors, some researchers have been interested in the process of Ferritin internalization in different cell types. They all agree that **Ferritin is internalized in a process of endocytosis mediated by vesicles coated with clathrin.**

Currently, there are few papers explaining the process of Ferritin endocytosis. Since 1987, the research group of Dr. Bligth studied the endocytic process of Ferritin in **guinea pig reticulocytes.** The same group in a previous work described the existence of a Ftbp in this cell type [46,56]. By using electron microscopy to detect Transferrin coupled to colloidal gold and native Ferritin, they observed that **Ferritin and Transferrin were internalized in the same covered vesicles.** After 60 min of incubation, both proteins were located in **large multi-vesicular endosomes.** These vesicles were then fused with the plasma membrane, **releasing Ferritin and Transferrin by exocytosis, both without iron.** 23-35% of Ferritin did not colocalize with Transferrin. This group suggests that Ferritin is internalized in the same way than Transferrin, namely by receptor-mediated endocytosis and by coated vesicles, but that these proteins are recycled only partly in common. Because Transferrin is a classic marker of endocytosis mediated by vesicles coated with clathrin, it makes us think that Ferritin is also internalized by endocytosis via clathrin [56].

Dr. Moss, in 1992, demonstrated the internalization of H-Ferritin in the human cell line of T lymphoid lineage (**MOLT-4**) and also described in this work an H-Ftbp [51]. This group, by testing the inhibition of Ferritin internalization, found that **chloroquine, which is a classic inhibitor of clathrin-mediated endocytosis,** inhibits H-Ferritin endocytosis in that cell line. This suggests that H-Ferritin is internalized through a mechanism of receptor-mediated endocytosis.

In 1994, this same group confirmed the previous results using confocal microscopy and electron microscopy. They discovered that H-Ferritin is internalized in these lymphoid cells and found that after 40 min of incubation, using fluoresceinated antibodies and a lysosomal marker coupled to rhodamine, **Ferritin reached lysosomes, in contrast to Transferrin, which was recycled.** By testing inhibition of endocytosis with chloroquine and observing with confocal microscopy, they ascertained that the Ferritin internalization was inhibited. These researchers did not conclude that Ferritin is endocytosed via clathrin-coated vesicles. Rather, since endocytosis of this protein can be inhibited with chloroquine, it is deducted that Ferritin is internalized by such vesicles [57].

As mentioned earlier, previous studies by Dr. Hulet have demonstrated the existence of FtRs in white matter of adult mouse brains and in human brains [53,54]. Because white matter is comprised mostly of oligodendrocytes, the same research group in the year 2000 reported that, **oligodendrocyte progenitor cells internalize Ferritin**. These researchers performed **endocytosis inhibition assays** evaluated by confocal microscopy and **saturation and competition assays.** They described the existence of a Ftbp. Additionally, using a **hypertonic media**, a **potassium deficient** media and another with **reduced ATP** (all inhibitors of clathrin-mediated endocytosis) they observed the inhibition of Ferritin endocytosis in these cells. Moreover, by **adding exogenous Ferritin**, there was a decrease in the binding of IRPs to IREs, indicating that Ferritin iron contributes to intracellular iron reserves. These researchers suggest that **Ferritin is an important iron source for oligodendrocytes**, providing 2,000 times more iron per molecule than Transferrin [58].

In Caco-2 cells, a human intestinal cell line, it was described by confocal microscopy and immunoassay, that these cells bind and endocytose soybean Ferritin. By competition assays they found that Ferritin binding is saturable, with a Kd = 7.75 ± 0.88 nmol/L, also the internalization of this protein is dependent on concentration, temperature and time. Finally, this group reported an endocytosis inhibition assay assessed by radioactivity and by confocal microscopy (using a fluoresceinated secondary antibody), where Caco-2 cells internalized ^{131}I-Ferritin and unmarked Ferritin through an endocytic process mediated by clathrin-covered vesicles. This group argues that Caco-2 cells utilize soybean Ferritin, using it as an iron source [59].

12. Ferritin and pathogens

Unfortunately, **Ferritin is not only an excellent iron source for our cells, but also for pathogenic and non-pathogenic microorganisms.** Unfortunately, in the literature the number of works about the use of Ferritin as a nutrient for bacteria and other microorganisms is limited. **As Ferritin is an intracellular protein,** pathogens must be capable of destroying cells and tissues to have access to this protein and make use of its iron. A recent review about Ferritin in its relationship with pathogens is found in [60].

Due to Ferritin is able to capture up to 4,500 iron atoms, this protein is really an abundant iron source for intracellular pathogens able to acquire its iron. Each pathogen has developed its own manner to obtain iron from Ferritin: for instance, the bacterium ***Neisseria meningitidis*** **triggers the host Ferritin redistribution from cytosol to lysosomes within infected epithelial cells and accelerates the Ferritin degradation by lysosomal proteases, thus providing the necessary iron for its own benefit [61].**

On the other hand, ***Burkholderia cenocepacia*** **secretes serine-proteases that degrade Ferritin [62].** Some bacterial species secrete siderophores, ultra-high affinity iron-binding compounds able to remove iron from the host Ferritin, such as some strains of ***Mycobacterium*** **[63].**

Another mechanism to get Ferritin iron is the reported in *Bacillus cereus* **and the mucosal pathogenic fungus** *Candida albicans,* **which possess** surface reductases **to acquire iron from Ferritin [64, 65].**

In parasitic protozoa practically there are no reports about the utilization of Ferritin as a sole iron source. *Trichomonas vaginalis* uses Ferritin iron but the mechanism by which uptakes iron from this protein is still unknown [66]. Our group has reported that the protozoan parasite that causes amoebiasis, ***Entamoeba histolytica*****, internalizes Ferritin through a process of endocytosis mediated by clathrin-covered vesicles, suggesting a similar mechanism of Ferritin endocytosis in this primitive eukaryote to that higher cells** [67]. This will be discussed in the chapter corresponding to this parasite.

Concluding, pathogenic microorganisms capable of removing and acquiring iron from Ferritin can obtain a plentiful source of this crucial metal to survive, colonize and invade the host.

12. Conclusion

Every cell requires iron to survive and to fulfill its essential metabolic functions. Iron excess is stored in an intracellular protein called Ferritin. Mammalian Ferritin is introduced into the cell through a mechanism of endocytosis via clathrin-covered vesicles. This process is mediated by a receptor on the cell surface.

13. References

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Chapter 9

Siderophores: What are they and how are they detected?

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1. Siderophores

Siderophores are **chelating agents**, synthesized by bacteria, fungi, and monocotyledonous plants in response to a low iron availability in the environment, although sometimes (as in mycobactins produced by mycobacteria) they are associated to cellular development [1].

Function of siderophores is **to take iron from the environment and carry it to the microbial cell**. Although most siderophores are soluble in water and are excreted, this does not occur in some of them (examples are the abovementioned mycobactins, which are localized inside the cell). In contrast with these, carboxymycobactins and exochelins are siderophores that pertain to the same bacterial group, but are localized extracellularlly [2, 3]. Extremely lipophilic siderophores have even been found in marine bacteria, as well as siderophores that do not actually diffuse to the environment but form vesicles [4]. Although distribution of siderophores in nature (based on the above statements) seems to be wide and their function is highly conserved.

In general, these iron transporting mechanisms are of non-destructive type, and **siderophores are reused**, although some can be degraded by **Esterases** after releasing the ferric ion to the cells [5].

2. Chemical structure of siderophores

Siderophores can be defined as **small peptidic molecules**, between 500 and 10 000 Da [6], rapidly assembled through short and well-defined metabolic pathways. These molecules contain lateral chains and functional groups that provide a high affinity (K_{aff} between 10^{-22} and 10^{-50} M) to coordinate with the ferric ion [7, 8].
Most siderophores contain one or more of the following bidentate ligands as construction blocks (Figure 1): i) dihydrobenzoic acid (**catecholate**) coupled to an amino acid; ii) **hydroxamate** groups with N^5-acyl-N^5-hydroxyornithine or N^6-acyl-N^6-hydroxylisine; iii) **hydroxycarboxylates** constituted by citric acid or β-hydroxyaspartic acid [5, 8].

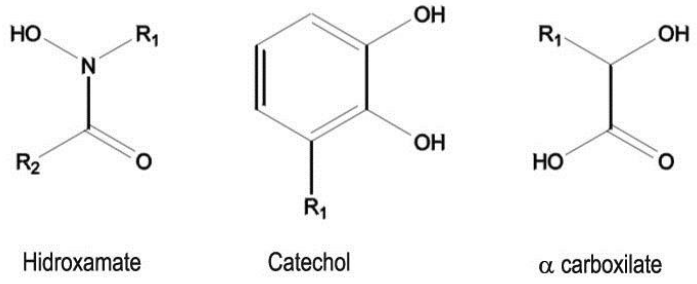

FIGURE 1. CHARACTERISTIC FUNCTIONAL GROUPS OF SIDEROPHORES.

The variety in the siderophores structure has been widened through the purification and characterization of new siderophores, including now molecules with **oxazoline, thiazoline, hydroxypiridinone, α and β hydroacid and α□-ketoacid groups**. The density of the ligand constitutes an important characteristic of siderophores, which cover **from bidentate to hexadentade chelants** [9, 10].

Although there is a great diversity in the structure of siderophores there are also many similarities, such as:

- **they contain strongly donating electron atoms (frequently oxygen and, to a lesser degree, nitrogen or sulfur),**
- **their shape is thermodynamically stable,**
- **they contain high spin Fe^{3+} species, and**
- **they have an oxide-reducing potential between -0.33 V (triacetylfusarinine) and -0.75 V (enterobactin)** [11, 12].

The preferred electronic configuration of Fe^{3+} in the Fe^{3+}/siderophore complexes is the *six-time coordinated octahedron*, in which one of the five **3d** orbitals is occupied by an electron. This geometry is relatively easy to twist, because the ferric ion has no directional preference due to the stabilization of the ligand's field. The arrangement undergone by the ferric ion when coupled is variable, it does not have a single possible position in space and this will depend on what the ion is coupled to.

At present, **more than 500 siderophores** have been chemically characterized and classified [5, 13].

3. Biosynthesis of siderophores

The biosynthesis pathways of siderophores are tightly linked to the **aerobic metabolism**, involving the use of molecular oxygen as activator and of mono- di- and N-oxygenases, as well as acids originated at the end of the citric acid cycle (as for example, citrate, succinate, and acetate). All siderophores of peptidic nature are synthesized by **non-Ribosomal synthetases** and, in the case of fungal siderophores; they are constructed mainly from **ornithine**, a non-protein amino acid [14]. In this way, synthesis of siderophores is mostly independent from primary metabolism [15].

4. Production of siderophores

Synthesis of siderophores is regulated by iron and is frequently influenced by other environmental factors. Hence, the growth conditions are important to maximize production of siderophores [16]. **The culture medium to be used must be treated to eliminate any iron traces (deferration) from it to avoid any**

possible repression in the synthesis of siderophores. Production of siderophores can be increased by favoring conditions in which the iron requirement by the cell is increased, or by reducing its availability. Examples of this are the use of carbon sources, such as **succinate** or **lactate** instead of glucose, by adding **manganese** to the culture medium, or by increasing **aeration** during the growth stage of the culture [17, 18].

Temperature of growth is also important, since production of siderophores can diminish with an increment in temperature, but this will generally depend on the optimal conditions of each microorganism.

In any case, an essential factor for the production of siderophores is the **pH of the culture**, which must be measured and adjusted during growth of the microorganism. Acid pH conditions increase iron solubility, reducing the production of siderophores, whereas high pH values could disrupt the molecules [19, 20].

Except for mycobactins, which are associated with the cell wall of the microorganisms that produce them [21], **siderophores are generally found in the supernatant of the culture**. Cells are normally precipitated by centrifugation, and the supernatant is subjected to any of the analyses used for siderophores detection.

5. Siderophore detection methods

Detection of siderophores can be as simple as using a qualitative or quantitative method that indicates their presence or as sophisticated as to need specific instrumentation according to the siderophore of interest. This type of assays allow obtaining a reliable response and, in general, a fast one for the detection of siderophores. In addition, it must be emphasized that **a positive result does not only indicate the presence of a siderophore but frequently also provides information about the functional group present.**

In the following, we will describe in detail some of the methods used to detect siderophores, **based on their chemical and/or biological features.** According to the former, assays are specific for the detection of hydroxamate, catechol, and α-carboxylate groups. Biological assays, in turn, are focused rather on working directly with the producing microorganisms than with the siderophore *per se*. Even so, the use of biological assays with the compounds of interest cannot be discarded in the absence of microorganisms.

5.1. Chemical assays

A) Hydroxamates

- *Tetrazolium test* [22]. This is a qualitative test to detect hydroxamates.

Procedure: One to two drops of 2 N NaOH and 1 mL of the sample to be tested are added to 20 mg of tetrazolium salt. If an intense red color appears immediately, it indicates the presence of a hydroxamate-type siderophore.

Reagents:

2,3,5-triphenyltetrazolium
2 N NaOH

- *$FeCl_3$ test* [7]. This is a qualitative test to detect siderophores, in general, and hydroxamates.

Many siderophores form **colored complexes in the presence of iron**. Therefore, some siderophores can be detected by the formation of this type of complexes when an iron salt is added to the culture medium.

Procedure: From 1 to 5 mL of a 2% ferric chloride solution are added to the filtered culture medium. Formation of red or purple color indicates the presence of siderophores; a peak in absorbance values of the iron-siderophore complex between 420 and 450 nm confirms that it is a hydroxomate-type siderophore.

Reagent:

2% $FeCl_3$ solution (w/v)

- *$Fe(ClO_4)_3$ assay* [23]. This is a qualitative test for the detection of siderophores.

Hydroxamates and other siderophores capable of forming stable complexes with iron at a low pH can be detected by means of the reaction with ferric perchlorate dissolved in perchloric acid. Catechols do not react in this assay because the iron is dissociated from these compounds at low pH values.

Procedure: A small amount (0.5 mL) of the culture supernatant is mixed with 2.5 mL of the ferric perchlorate reagent. The formed complexes usually depict colors between orange and royal purple.

Reagent:

5 mM $Fe(ClO_4)_3$ in 0.1 M $HClO_4$

- *Csáky* assay [24]. This is a qualitative test to detect hydroxamates.

This assay is more sensitive that that of perchlorate, although still more qualitative than quantitative. The Csáky assay detects the presence of **secondary hydroxamates** and depends on the oxidation to nitrite and on the formation of a colored complex through a diazonium-type bond.

Procedure: One milliliter of the culture supernatant is hydrolyzed with 1 mL of 6 N H_2SO_4 in a hot water bath during 6 h (or at 130 ºC during 30 min). The solution is buffered with 3 mL sodium acetate and, then, 1 mL of sulfanilic acid followed by 0.5 mL of the iodide solution is added. After incubating at room temperature during 3 to 5 min, the excess iodide is destroyed with 1 mL of sodium arseniate. Then, 1 mL of an α-

naphthylamine solution is added and the volume is brought up to 10 mL, allowing for the change in color during 20 to 30 min. Absorbance of the reaction is determined at 526 nm.

Reagents:

6 N H_2SO_4
Sulfanilic acid: 1 g of sulfanilic acid is dissolved by heating in 30% (v/v) acetic acid.
Iodide solution: 1.3 g of iodide is dissolved in 100 mL of glacial acetic acid. Sodium arseniate: 2 g of Na_3AsO_4 are dissolved in 100 mL of distilled water.
Sodium acetate: 35 g of sodium acetate are dissolved in 100 mL of distilled water.
α-naphthylamine: 3 g of α-naphtylamine are dissolved in 1 L of 30% (v/v) acetic acid.

B) Catechols

- *Arnow* assay [25].

Procedure: To 1 mL of supernatant (or of the siderophore in solution), 0.1 mL of HCl and 1 mL of Arnow's reagent are added, until a yellow color is observed. Afterwards, 1 mL of NaOH are added and the color changes to red, adjusting the volume to 5 mL. The color is stable for at least 1 h, and the solution has a maximal absorbance at 515 nm.

Reagents:

5 N HCl
1 N NaOH
Arnow's reagent: 10 g of $NaNO_2$ and 10 g of $Na_2MoO_4 \bullet 2H_2O$ are dissolved in 50 mL of Milli Q-quality water.

- *$FeCl_3$* assay [7].

Procedure: This method has already been described under the previous section, for the detection of hydroxamates. The red or purple color indicates the presence of siderophores, and a peak of 495 nm in absorbance values of the iron-siderophore complex confirms that the latter is of the catechol type.

C) Carboxylates

- Shenker's test [26]

Procedure: To 1 mL of the filtered culture medium, 1 mL of 250 μM $CuSO_4$ and 2 mL of the acetate buffer, pH 4, are added. The absorbance of the resulting copper-siderophore complex is analyzed between 190 and 280 nm.

Reagents:

250 μM $CuSO_4$
Acetate buffer, pH 4

5.2. Biological assays

Although the previously described assays are based on the chemical properties of siderophores, some compounds cannot be detected with these methods. The assays that depend on the biological or functional properties are, quite often, more sensitive to the presence of siderophores, providing results as reliable as those obtained with a chemical assay. Methods for the detection of siderophores, in general, have been developed, and the most used ones are based on the use of the **chrome-azurol S** compound.

A) Chrome azurol S (CAS) universal assay

The ternary complex CAS/Fe^{3+}/HDTMA is the one that functions as indicator of siderophores: when a strong chelating agent takes up the Fe^{3+} from the indicator, the latter changes its blue color to orange [27]. There are two variants of this assay: the CAS-agar plate and the liquid assay.

- CAS-agar plate assay

This assay is developed on top of the culture medium that incorporates the indicator, for this reason it can only be used to detect siderophores produced by Gram negative bacteria (the hexadecyltrimethylammonium, HDTMA, present in the medium is a growth inhibitor for any other type of microorganism).

Procedure: A mixture of 100 mL of salts 10× MM9, 15 g of bacteriological agar, 30.24 g of PIPES, and 750 mL de H_2Od, adjusted to a pH of 6.8 is sterilized in an autoclave. In addition, 605 mg of chrome azurol S are dissolved in 500 mL of Milli Q-quality H_2O and mixed with 100 mL of a 1 mM $FeCl_3$•$6H_2O$ in 10 mM HCl solution. This solution is added under agitation and slowly to 729 mg of HDTMA dissolved in 400 mL of Milli Q-quality water, obtaining a dark blue mixture that is also sterilized in an autoclave.

The solution with the agar is cooled to 50 ºC, then 30 mL of iron-free casaminoacids are added. For bacterial growth, a carbon source is needed (for example, 10 mL of 20% glucose in water) with other supplements (1 mL of 1 M $MgCl_2$ and 1 mL of 100 mM $CaCl_2$, for example. If needed, vitamins and antibiotics can be added), All these compounds are added as sterile solutions.

Finally, the CAS/$FeCl_3$/HDTMA solution is slowly added running down on the wall of the container, mixing and avoiding foam formation; 30 mL of the final mixture are poured into Petri plates, the intended strains to be studied can be sown once the medium solidifies. After incubating under adequate conditions (temperature and time will depend on the microorganisms of interest), a color change will occur in the medium if a siderophores producing bacterium developed on it.

Reagents:

Salts 10× MM9: 3 g of KH_2PO_4, 5 g of NaCl, and 10 g of NH_4Cl are dissolved in 1 L of Milli Q-quality water.
Bacteriological agar
PIPES
H_2Od and H_2O (Milli Q-quality)
Chrome azurol S (CAS)
1 mM $FeCl_3 \cdot 6H_2O$
10 mM HCl
HDTMA
Casaminoacids iron-free solution: 10 g of casaminoacids are dissolved in 100 mL of H_2Od.
Glucose or carbon source
$MgCl_2$
$CaCl_2$
Vitamins and supplements, if required.

- *Liquid assay*

Procedure: 0.5 mL of CAS solution and 0.5 mL of filtered culture medium or of the siderophores sample are mixed, adding 10 µL of S solution, leaving it to rest for 1 h and the absorbance of the mixture is measured at 630 nm. The reading of the sample must be lower than the reading of the reference. Concentration of siderophores is calculated with the following formula:

$$\textbf{\% siderophores} = [(Ar - As)/Ar] \times 100$$

Where: **Ar** is the reading of the reference and **As** is the reading of the siderophores sample.

Reagents:
CAS solution: 1.5 mL of 1 mM Fe stock solution and 7.5 mL of CAS stock solution are mixed and added to the HDTMA solution. Finally, the piperazine buffer is added and brought to a 100 mL volume with Milli Q-quality H_2O.
Stock solution 1 mM Fe: 1 mM $FeCl_3 \cdot 6H_2O$ dissolved in 10 mM HCl.
Stock solution 2 mM CAS: 0.121 g of chrome azurol S dissolved in 100 mL of H_2Od.
HDTMA solution: 0.0219 g of HDTMA dissolved in 50 mL of H_2Od.
Piperazine buffer: 4.307 g of piperazine are dissolved in 30 mL of H_2Od, adjusting the pH to 5.6 with concentrated HCl.
S solution: 0.2 M sulfosalicylic acid; stored in darkness.

B) Modified CAS assay

This assay is also based on the ternary CAS indicator complex. The objective of the authors [28] with this modification was to avoid the inhibition produced by HDTMA on microbial growth and to permit the detection of siderophores produced by any type of microorganisms. In this case, the Petri dish is divided in two halves: in one, CAS agar is placed (as in the afore described technique), placing, in the other half, the solid medium required by the microorganism of interest. When the microorganism grows in this second half, siderophores diffuse, reaching the other half of the Petri dish, containing the CAS agar, and modify its color.

In this way, inhibition of growth is avoided, allowing for an easy detection of the produced siderophores by different organisms, including Gram negative bacteria and fungi. However, this assay has the disadvantage of being only qualitative. Besides, preparing the two halves of the Petri dish is somewhat cumbersome.

Procedure: The CAS agar is prepared according to the Schwyn and Neilands methodology [27], previously described. The Petri dishes (10 mm in diameter) are prepared with 30 mL of the culture most appropriate for each microbial strain to be studied. Once the medium has solidified, one of the halves is replaced by CAS agar (15 mL). The half containing the culture medium is inoculated with the strain being studied, sowing as far as possible from the line dividing the media. Incubation is made at the temperature adequate for the growth of each studied microorganism.

Reagents:
These are the same as those descibed under A, in the modality of assay in solid medium, except that, in this case, neither salts 10× MM9, iron-free casaminoacids, carbon source, nor the other supplements are needed for bacterial growth. In this modality, growth takes place in the second medium added to the Petri dish.

C) O-CAS assay

Our objective with this method was to design and optimize a specific assay for the detection of siderophores in a solid medium [29]. The traditionally used assay (CAS), based on the use of chrome azurol S and with the inconveniences already mentioned, was redesigned to obtain a new, fast, non toxic, easy to use method that could be instrumental for the detection of a large variety of siderophore-producing microorganisms. The assay allows also for the identification of more than one producing microorganism at the same time, because the revealing medium is overlaid on the medium (hence, its name O-CAS) once the producing microorganisms have developed on the culture medium most appropriate to their characteristics.

Procedure: For 1 L of O-CAS, 72.9 mg of HDTM are dissolved in 40 mL of Milli Q-quality H_2O, avoiding foam formation. In a separate container, 60.5 mg of CAS are dissolved in 50 mL of Milli Q-quality H_2O, adding 10 mL of 1 mM $FeCl_3 \bullet 6H_2O$ dissolved in 10 mM HCl. The color of the solution intensifies at this moment. This CAS solution is added on top of the HTDM solution and the color changes to blue. In a separate container, 30.24 g of PIPES are dissolved in 800 mL of Milli Q-guality H_2O, and the pH is adjusted to 6.8, adding agarose afterwards.

The two solutions are sterilized separately and mixed only when a temperature of approximately 55 ºC is reached, avoiding gelification of the medium and, thereby, iron precipitation.

Once microorganisms, to be tested, have been incubated in the optimal medium for their development (it is very important to free them from iron before [30]) the O-CAS is overlaid on them (10 mL of medium for

standard 90-mm diameter plates, and 30 mL of medium for 150-mm diameter plates). The overlay is left to gelify and results can be observed after 15 min.

The following organisms can be used as controls: *Aureobacterium flavescens* JG9 for the detection of hydroxamates, *Rhizopus oligosporus* ATCC 22959 for carboxylates, and *Aspergillus niger* ATCC 66876 for the detection of catechols.

Reagents:
HDTMA, 72.9 mg
Chrome Azurol S (CAS), 60.5 mg
PIPES , 30.24 g
1 mM $FeCl_3 \cdot 6H_2O$ in 10 mM HCl, 10 mL
Agarose, 0.9% (w/v)

5.3. Other biological assays

There are other assays developed to solve specific questions, as for example the Ames-Gottfred *et al*. method [17] , in which different carbon and nitrogen sources are used to improve siderophores production. Calvente *et al*. [31] developed an assay to detect siderophores specifically produced by yeasts. Renshaw *et al*. [32], on their part, developed a method to detect UO_2^{2+}, aside from investigating the capacity of fungal metabolites to form complexes with actinic compounds.

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Chapter 10

Iron transport across the bacterial surface

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Abstract

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Abstract

In bacteria, and especially pathogenic bacteria, iron is an essential element for their survival and multiplication. These microorganisms have developed one or more iron uptake systems able to compete with the iron natural carriers and the storage mechanisms of the host. In some cases, this competition determines the ability of bacteria to successfully colonize the tissues.

Bacterial infection is a multifactorial complex process, which involves a dynamic struggle between the host defense mechanisms and a variety of microbial virulence factors, such as the struggle for iron. Although the host tissues are relatively rich in iron, most of it is intracellular**.** Free iron in our body is quickly chelated by proteins such as Transferrin and Lactoferrin, thus obstructing the availability of this element for bacteria.

Experimental evidence shows that the ability of some pathogenic bacteria to internalize iron promotes the expression of virulence factors that allow them to survive under physiological conditions. Currently, it is known that iron has a regulatory effect on the expression of genes involved in iron uptake, as well as other genes encoding factors related to bacterial virulence. These genes have been included as virulence markers in molecular epidemiology studies.

This chapter lists some of the mechanisms of iron uptake and their relationship with bacterial virulence.

1. Introduction

Iron is an essential nutrient for all organisms due to its critical role in diverse biological processes. **Bacteria need an iron concentration between 10^{-6} and 10^{-8} M, except some acid lactic bacteria, which do not need of iron** [1].

In both mammals and microorganisms iron is directly involved as a **cofactor** in the Ribonucleotide reductase, RNA polymerase III, Hydrolases and Dehydrogenases, as well as in the **oxygen metabolism** enzymes: Superoxide dismutase, Catalase and Peroxidase; and in the **electron transference enzymes, such as:** Cytochromes, Ferredoxin, and Succinate dehydrogenase. Besides, in mammals **the activation of natural killer (NK) cells, T and B lymphocytes, and the neutrophils function all are dependent on iron** [2].

The **Fe^{2+} / Fe^{3+}** pair has a **redox potential** range from -300 mV to 700 mV, based on the ligand and protein group. Free iron **reacts with hydrogen peroxide by the Fenton reaction and produces hydroxyl radicals.** This biocatalytic property and its wide reactive capacity promote the generation of reactive oxygen species that damage DNA, the lipidic membranes, proteins and other cellular components [3].

Bacterial infection is a complex and multifactorial process that involves a dynamic competition between host defense mechanisms (both innate and acquired immunity), and a variety of microbial virulence factors that permits the pathogens to survive in the hostile environment of the host tissues. **During**

colonization, bacteria must obtain nutrients to proliferate. In this process iron plays an important role in the establishment and progress of the infection.

However, even when the host tissues and fluids are relatively rich in iron, most of it is intracellular or associated in other complex forms (such as **Hemoglobin** or **Ferritin**), and is thus not available to bacteria. **Extracellular iron is bound by iron-binding proteins with a high affinity such as Transferrin and Lactoferrin**, which normally are partially iron-saturated. Bacteria must get the necessary iron to survive and replicate. Thus, successful pathogens have one or more efficient iron uptake systems that allow them to compete with the carriers and iron storage mechanisms of the host [4].

2. Iron Uptake Systems in Bacteria

2.1. Siderophores

In previous chapters of this book, the characteristics of iron chelating systems have been reviewed; these systems compete with both Lactoferrin and Transferrin for iron. In this chapter we now refer to the association between iron uptake mechanisms and bacterial virulence.

It is important to remember that the **iron uptake mechanisms of pathogenic bacteria** (both Gram negative and Gram positive bacteria) have been classified in two types. The **first type** consists of **siderophores:** iron chelants defined as low molecular mass molecules with high affinity for iron, which compete with lactoferrin and transferrin for this metal. There is a wide diversity of siderophores with varying degrees of effectiveness.

Siderophores mainly include catecholates, hydroxamates and hydroxycarboxylates [5], while the associated mechanism involves their synthesis and release outside the cell. Some outer membrane proteins act as iron-siderophore receptors and are essential for the uptake of this element. **The second type** of iron uptake mechanism includes **Receptors to iron-containing proteins, such as Transferrin, Lactoferrin, Ferritin and Hemoglobin:** these **receptors** are proteins that maintain a direct interaction with the corresponding host iron proteins [6].

Siderophores have specific receptors, which are dependent on the TonB energy system. Examples of these receptors in ***Escherichia coli*** include: **Btu for vitamin B12 and six iron receptors (FhuE, FhuA, FepA, FecA, Cir, and Fiu)**. These proteins bind their ligands with high affinity (Ka) even in the nanomolar range [7].

FepA is an 81-kDa protein that recognizes the iron-charged siderophore **Fe-enterobactin: a tricatecholate compound** that holds an extremely high affinity for Fe^{3+} ions. It captures exogenous Fe^{3+} by forming a complete six-ligand coordination sphere around the element. Once FepA binds Fe-enterobactin, it imports this ferric siderophore into the periplasm. FepA is composed of a 22-stranded membrane-spanning β-

barrel with a globular N-terminal plug domain of 148 residues that folds up inside the barrel. This domain features a predominantly unfolded state, which binds ferric enterobactin and also some antibiotics that penetrate into this region. FepA has been isolated from ***E. coli*** and ***Salmonella enterica*** **serovar Typhimurium** [8].

FhuA (78 kDa) **is a trihydroxamate siderophore receptor for ferrichrome uptake.** It is produced by several fungal species, including ***Ustilago sphaerogena*** **and all species of** ***Penicillium*** [2]. Structural studies of this receptor in *E. coli* (**Figure 1**) showed a β barrel composed of 22 antiparallel β strands (residues 160-714). **In contrast to the typical trimeric arrangement found in porins, FhuA is monomeric.** Located within the β barrel, there is a structurally distinct domain, the "cork", which mainly consists of four β-stranded sheets and four short α helices. The FhuA amino acid sequence is adapted for the ferrichrome uptake. In addition, its fourth fold is used as a **receptor by the T1, T5, and φ80 bacteriophages** [9].

FhuE is the receptor for ferric-coprogen and ferric-rhodotorulic acid siderophores. This receptor is mainly produced by ***Penicillium chrisogenum*** (penicillin-producing fungus) and ***Neurospora crassa*** (bread mold, extensively studied at genetic level). When the *fhu*E gene was expressed in **minicells**, it was observed an **FhuE precursor protein** of 82 kDa and its final form of 76 kDa [10]. The precursor contained a signal peptide of 36 amino acids long. The amino-terminal end of the mature form contained the sequence Glu-Thr-Ile-Val and two uncharged residues ending with Val. **This final sequence was found in all outer membrane receptor proteins that were constituents of the TonB-dependent transport systems.**

Cir and Fiu are two proteins in *E. coli*, which confer susceptibility to catechol substitutes, β-lactams and cephalosporins. This suggests an important physiological role in the **transport of the iron-monocatecholic complex, in addition to the uptake of enterobactin** hydrolytic products, such as 2-3 dihydroxy-benzoic acid and the 2-3 dihydroxy-benzyl-serine [11].

FecA is an outer membrane protein which is derepressed in response to iron limitation in the presence of 0.1 mM citrate. This protein **is required for the use of ferric dicitrate as an iron source**. Citrate may not be considered a true siderophore, since it is only effective in high-level concentration and the derepression of the protein requires that this molecule be present in the extracellular environment. FecA is a monomeric β-barrel receptor composed of 22 strands with an N-terminus from 150 to 200 residues. A globular domain was found inserted in the barrel at periplasmic side forming a plug [12].

In iron-limiting conditions, bacteria synthesize siderophores of two different chemical groups: aerobactin (hydroxamate type) and enterobactin (phenolate type). The **aerobactin** system is derepressed under stress conditions due to a lack of iron, and **is associated with a new outer membrane protein called LutA, similar to Cir in electrophoretic mobility** (74 kDa). Investigations show that the aerobactin system is an **operon** composed of 5 genes, four are involved in aerobactin **biosynthesis and the fifth encodes the 74 kDa outer membrane protein that serves as the receptor for ferric-aerobactin**. Interestingly, a**erobactin**

may be more efficient than **enterobactin** in competing for iron against host proteins. One reason to consider this is that enterobactin is bound to albumin and is consequently inactivated. In contrast, aerobactin is not bound to albumin and thus enables bacteria to obtain iron expeditiously.

Iron uptake systems mediated by siderophores have likewise been reported in Gram-positive bacteria. ***Mycobacterium*** species are intracellular bacterial pathogens, and synthesize two types of iron binding compounds: **mycobactin** and **hexochelin**. Hexochelins are soluble and solubilize iron to transport it into the cell. The structure of these compounds is practically unknown, aside from the fact that they contain **N-hydroxy-ornithine** as the chelating agent. Mycobactins are hydrophobic and remain attached within the cell wall where act as storage compounds [13].

A siderophore-like activity has been detected in ***Corynebacterium diphtheriae*** [14], ***Staphylococcus aureus*** and ***Staphylococcus epidermidis***, which use an enterochelin-iron complex to produce their own siderophores. Furthermore, in ***Staphylococcus hyicus,*** **staphyloferrins A and B** (siderophores with ornithine and two citric acid residues) were isolated [15].

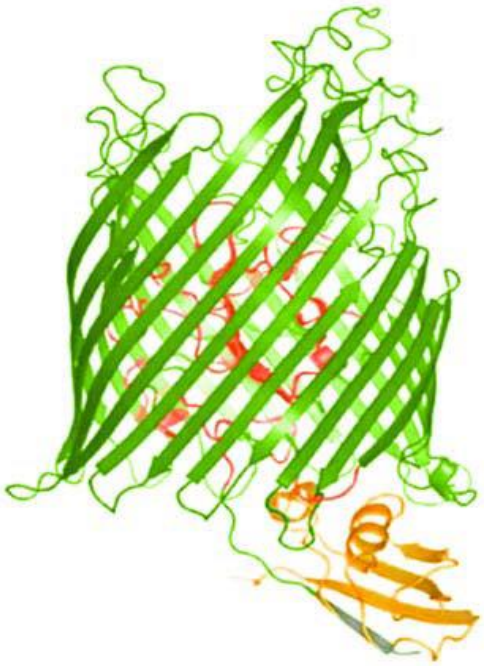

Figure 1. TRIDIMENTIONAL STRUCTURE OF THE OUTER MEMBRANE PROTEIN FhuA [9] (drawn by netxos.com)

2.2. Iron transport systems independent on siderophores

Little is known about iron uptake systems that are not dependent on siderophores. The reader is advised that an extensive discussion on this topic can be found in **other chapters of this book.**

In addition to siderophores, another strategy for iron uptake is used in both Gram-negative and Gram-positive bacteria through an independent chelating agent pathway. **Ferrous and ferric iron can be directly imported by host iron complexes (Transferrin, Lactoferrin, Heme proteins, etc.) and iron storage proteins (Ferritin).** Bacteria control their iron requirements in response to iron availability by down regulating the expression of iron proteins during iron-restricted conditions. The expression of the iron homeostatic machinery controls the iron acquisition, storage and consumption to iron availability and thus intracellular level of free iron does not reach toxic levels [4].

Transferrin (Tf) is an extracellular iron-binding glycoprotein, **located in the serum and lymphatic system, where it is partially saturated with Fe^{3+} (holoTf).** Tf has a Mr that ranges from 75 to 80 kDa and provides iron to a wide variety of cells. Moreover, it acts like a bacteriostatic agent and also binds iron tightly but reversibly. Although iron bound to Tf accounts for less that 0.1% (4 mg) of the total iron body, it is the most important iron pool with the highest rate of turnover (25 mg/24 h).

The Tf molecule has two specific high-affinity Fe(III) binding sites. Likewise, the affinity of Tf for Fe(III) is high (10^{-23} M at pH 7.4). The heterodimeric receptor complex is composed of two proteins, Tf-binding protein A (TbpA) and Tf-binding protein B (TbpB). The **Transferrin receptor (TfR) is a functional complex of three proteins.** One carries iron from the outer membrane into the cytoplasmic membrane, whereas the other two proteins are involved in Tf binding and subsequent iron release. Tf is transported into the cell in a vesicle by receptor-mediated endocytosis [16].

Ferritin is a protein of 450 kDa consisting of 24 subunits. Each Ferritin complex can store up to 4500 iron ions (Fe^{3+}). Ferritin is a soluble intracellular protein that both stores and releases iron. Likewise, the protein is formed by two components: **an iron micelle of hydroxide phosphate $(FeOOH)^3$ $(FeOOP_3OH_2)$** and **a multi-subunit protein called apoFerritin** [16]. Within cells, furthermore, iron is stored in Ferritin.

Hemoglobin is another intracellular iron-containing protein, which transports oxygen in the blood cells and consists of two pairs of polypeptide chains: α and β. Moreover, Heme residues are located in a hydrophobic box in each string. The total Mr is about 64.45 kDa [16].

Interestingly, **neither *Neisseria gonorrhoeae*** (bacterial causal agent of gonorrhea: a sexually transmitted disease) **nor *Haemophilus influenzae*** (bacterial pathogen that causes pneumonia, especially in children under 5 years), **produce siderophores**. *H. influenzae* **is able to exclusively use human holoTransferrin** as an iron source, while the ***Neisseria*** **species can use both human holoLf and holoTf proteins** [17]

Other microorganisms such as ***Mycoplasma pneumoniae,*** which causes atypical pneumonia and the ***Trichomonas vaginalis*** protozoan, which parasitizes the urogenital tract, have specific receptors for iron-charged Lactoferrin (holoLf). Likewise, ***Bordetella pertussis*** (the agent of whooping cough) produces siderophores of the hydroxamate type, while it uses holoTf and holoLf concurrently as iron sources. Although in the latter case, the use of siderophores is a more effective iron uptake system than holoTf [17].

In spite of the unavailability due to its intracellular location, Heme group is known to be an iron source for microorganisms. *In vivo* studies on ***H. influenzae*** have shown instances of iron uptake from Heme compounds complexed to **Hemopexin**, serum albumin, and, on the other hand, **Hemoglobin bound to Haptoglobin.** These studies suggest that, **in the intracellular environment, iron is preferentially acquired from the Heme compounds**. This is an advantageous strategy of intracellular bacteria such as the ***Shigella*** species (the major cause of bacterial dysentery), which are able to synthesize siderophores, and once established in the epithelium, they acquire intracellular iron through Heme compounds.

Vibrio species internalize iron from Heme compounds and interestingly **use neither holoLf nor holoTf for growth.** These bacteria **produce iron-regulated hemolysins;** hence their infections are characterized by an increase of free Hemoglobin. ***In vitro* studies of *V. cholerae*** (a cause for cholera pandemics) have shown that **Hemin and Hemoglobin are both used as exclusive iron sources** [17].

All the microorganisms mentioned previously produce **Transferrin Receptors (TfRs)** capable of binding iron as the first step in this intricate uptake system. The bacterial heterodimeric outer-membrane TfR is composed of two proteins, **TbpA and TbpB.** TbpB is a hydrophilic molecule attached to a small lipid domain in the outer membrane that not only recognizes iron complexed to Tf, but also presents it to TbpA. Additionally, **TbpA (a dimeric protein) internalizes Fe(III) into the cell by an ATP binding transport system (ABC). TbpA is proposed to form a β-barrel and to function as a gated porin** [16]. The ABC transport system is formed by **FbpA** (an Fe(III) binding protein), **FbpB** (an inner membrane protein) and **FpbC** (a cytoplasmic ATPase) [18].

The molecular mechanisms of iron transport in G**ram positive** bacteria are less known. In ***Staphylococcus*** species, investigations have revealed that only a small number of iron chelants are enough for growth. Moreover, ***S. aureus*** and ***S. epidermidis*** are able to take up iron from holoTf. In these species, the **TfR** has been named **Tpn**, a binding protein of 42 kDa located in the cell wall [19].

In **Gram-negative** bacteria, ions and nutrients are transported across the cytoplasmic membrane via a high-affinity energy-dependent process [20]. The high affinity iron uptake in *E. coli* is a process that involves:

1) the recognition of an iron chelant by a receptor in the outer membrane,

2) the translocation of the siderophore-Fe complex (III) to periplasm and

3) the iron internalization by a permease in the cytoplasmic membrane [18].

2.3. TonB energy system.

The study of energy requirements for acquisition of iron and other compounds showed the importance of the **TonB ExbB/D protein complex**. For example, the transport of **ferrichrome** and the phage T1 and φ80 infection occurs by an energy dependent process [21,22]. **The energy is derived from an electrochemical proton gradient generated by the electron transfer from the cytoplasmic membrane** (**Figure 2**).

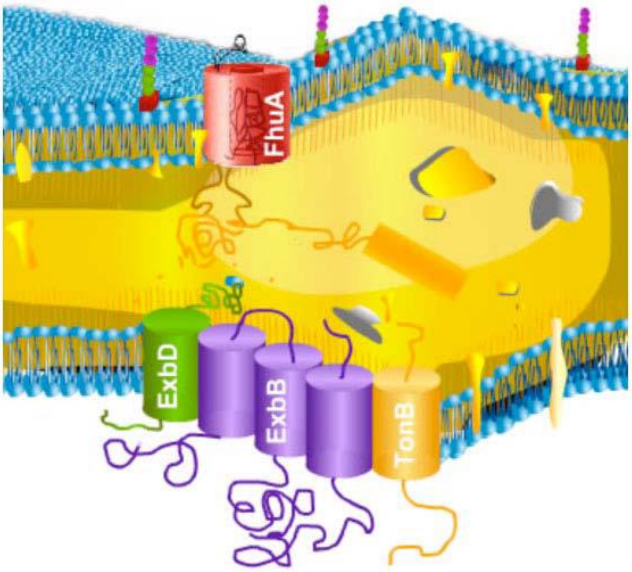

Figure 2. MODEL OF THE OUTER MEMBRANE PROTEIN FhuA AND TonB (ExbD, ExbB, TonB) ENERGY SYSTEM (drawn by netxos.com)

The TonB system consists of three proteins: ExbB, ExbD and TonB. **ExbB has three transmembranal domains** with an N-terminus that slightly twists near the periplasm. **ExbD is anchored to the cytoplasmic membrane** by the N-terminus **and the periplasmic protein TonB** has a conserved domain binding FhuA [17]. All of the TonB-dependent receptors have an N-terminal short sequence called **TonB box**. **The TonB complex distends the periplasm and interacts with the siderophore receptors, resulting in energy transduction by a common mechanism in TonB-dependent receptors.**

TonB and its homologues have been identified in various Gram negative bacteria such as ***S. enterica* serovar Typhimurium**, ***Yersinia enterocolitica***, ***H. influenzae*** and ***Pseudomonas aeruginosa*** [17]. ***V. cholerae* possesses a TonB-ExbB-ExbD system with more constituents**, as shown by the presence of the proteins **TonB1** and **TonB2**, which seem to have different specificity for outer membrane receptors. However, the reason for this dichotomy remains unclear [23].

In *E. coli,* the siderophores complexed to iron (Fe[III]) or ferrichrome are imported through the FhuA outer membrane receptor activated by the TonB energy system. They are also attached to the FhuD periplasmic binding protein, which is internalized into the cytoplasm by FhuB and FhuC proteins (**Figure 3**) [24]. The FhuB, FhuC and FhuD proteins are components of an energy-dependent **ABC transporter**.

In ***Actinobacillus pleuropneumoniae*** (bacterium that causes swine pleuropneumonia), it has been shown that the iron internalization from the outer membrane into the cytoplasm **needs the TonB ExbB-ExbD**

energy transduction system. Comparatively, in ***Haemophilus parasuis*** (which produces a respiratory disease in pigs), the presence of ***tonB***, ***exbB***, ***exbD***, ***tbpB*** and ***tbpA*** genes has been demonstrated in all the serotypes by PCR assays. Furthermore, all these genes share the same size as the gene in *A. pleuropneumoniae* and are arrayed consecutively in the bacterial chromosome.

Likewise, in ***Pasteurella multocida*** (a respiratory pathogen of pigs and other animals, including humans), ***Mannheimia haemolytica*** (a respiratory pathogen of ruminants) and *H. influenzae*, the gene arrangement is different, the *exbB* gene located in the first place, followed by *exbD* and finally *tonB*. The position where *tbpB* and *tbpA* are localized after the *exbD* genes in *H. parasuis*, has only been observed in *A. pleuropneumoniae* and *Actinobacillus suis* [25]. The last four genera mentioned belong to the *Pasteurellaceae* family.

2.4. ABC transporters

ABC transporters are transmembranal proteins that use energy from the hydrolysis of adenosine triphosphate (ATP) to carry out certain biological processes including the translocation of various substrates through the membrane [26].

The ABC transporter system consists of several components: **a) periplasmic proteins that capture the substrate with high affinity, b) a heterodimer composed of two integral membrane proteins** (each one with 5 or 6 α-helices that cross the cytoplasmic membrane) called permeases, responsible for conveying the substrate to the cytoplasmic membrane proteins; and c**) two peripheral membrane proteins that possess an evolutionarily conserved cytoplasmic domain** (200 amino acids), **called ATP-binding cassette. This, in turn, is attached to the cytoplasmic side, including the conserved ABC module that couples the hydrolysis of ATP** [26].

The mechanism of transport begins with the **binding of the substrate by the periplasmic protein.** The binding then induces a **conformational change that is recognized by the permease**. Subsequently, the permease **removes the ligand and transports it across the cytoplasmic membrane** using the energy provided by the **ATP hydrolysis**. Next, the periplasmic protein sends a signal to the hydrolase to avoid unnecessary consumption of ATP [27].

2.5 The Prokaryotic ABC proteins

ABC bacterial transporters are essential for viability of bacteria as well as for their virulence and pathogenicity [28]. For example, the ***chvE*** gene in ***Agrobacterium tumefaciens*** (a bacterium that causes tumors in plants by transformation), **encodes for importers of glucose and galactose** which have been associated with its virulence [29]. Another example is the role of the regulatory systems that neutralize the undesired changes in the cell. For instance, t**he increase of the osmotic strength is regulated by the activation of the osmo-sensibility of the ABC transporters that mediate solute uptake** [30].

Another associated function of the ABC transporter proteins **is the regulation of a large number of physiological processes** [28], such as the **bacterial efflux systems**. These systems remove or expel certain substances like surface components (e.g. **lipopolysaccharides, capsular polysaccharides, and teichoic acids**), proteins involved in bacterial pathogenesis (**hemolysins, iron-binding proteins and alkaline proteases**), **heme groups, hydrolytic enzymes, S-layer proteins, competitive factors, toxins, antibiotics, bacteriocins, peptide antibiotics, medicines (drugs) and siderophores** [31]. Similarly, ABC transport proteins play an important role in certain biosynthetic pathways, for example, the **extracellular polysaccharide biosynthesis** [32] and **the cytochrome biogenesis** [33].

One of the first iron ABC transporters characterized was the **Sfu system of *Serratia marcescens*** [34]. Additionally, **in the case of *Neisseria***, its iron transport system is formed by three proteins: **FbpA**, **FbpB** and **FbpC**. FbpA is the Fe(III) binding protein; this is the initial acceptor. FbpB is a cytoplasmic permease, and FbpC is an ATPase [35]. FbpC is the last protein that interacts with FbpA and FbpB forming, altogether, a typical ABC transporter.

FhuBCD, an ATP-dependent hydroxamate transport system, is a member of the superfamily of ABC transporters. FhuBCD complex catalyzes iron transport across the inner membrane into the cytoplasm of *E. coli*. Based on their sequence, it can be predicted that FhuB is a transmembranal component; FhuC is an ATP-binding subunit, while FhuD is a siderophore-binding periplasmic component [20].

The ABC systems in Gram-positive bacteria are less studied. In general, they resemble their Gram-negative counterparts, except that **they lack periplasmic transporters.** Instead, they utilize an analogue protein that also scavenges the micronutrient from the outside. This protein is anchored in the outer side of the cytoplasmic membrane, near the integral membrane heterodimer.

3. Iron uptake systems and bacterial virulence

Although iron levels in living organisms are in low requirements, it is possible that **possessing systems with high affinity for host-iron can be an important component of bacterial virulence**. It has been observed that **the presence of siderophores in strains of *Salmonella, Vibrio vulnificus, Yersinia* and *Pseudomonas* correlated with a markedly elevated virulence** [2].

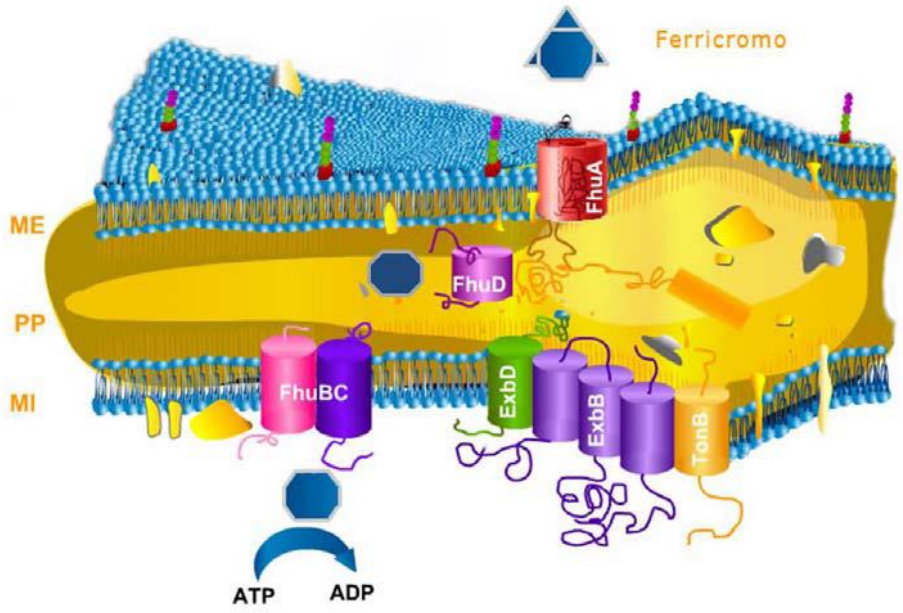

Figure 3. IRON UPTAKE SYSTEM BY FhuA, TonB AND FhuBCD. (OM) OUTER MEMBRANE; (PP) PERIPLASM; (IM) INNER MEMBRANE. (drawn by netxos.com)

A similar observation is the presence of **aerobactin system** for iron uptake, which is **more prevalent in *E. coli* strains that cause intestinal infections in humans and animals.** Even though this does not imply that iron uptake systems contribute to virulence, it is important to realize that each system plays an important role in host-parasite interaction [2].

3.1 Iron-regulated Outer Membrane Proteins (IROMPs)

The iron-regulated outer membrane proteins (IROMPs) usually serve as **receptors for iron-siderophore complexes and facilitate their internalization**. These high-affinity iron transport systems have been found in various bacterial pathogens [36,37,38,39,40,41]. Little is known about iron response systems in bacteria independent of siderophore synthesis. There is information on iron uptake from serum (**Heme**) proteins. The mechanism of the Heme-iron system response and its possible role for outer membrane proteins is not entirely clear [42,43,44,45].

Heme is a tetrapyrrole molecule which binds iron and an essential component in many biologically active molecules. To enumerate them, **Hemoglobin, Myoglobin, Cytochromes, Catalases and Peroxidases** all contain Heme as a prosthetic group. The biological utility of the Heme group depends on Fe availability to alternate between its two oxidation states: Fe(II) and Fe(III).

Due to the toxic nature of the Heme group (especially when complexed to Fe[III]), it cannot circulate freely within the host. When the molecules containing Heme groups are degraded, e.g. by metabolic natural processes or an eventual damage, the released Heme groups are quickly surrounded by molecules such as **Hemopexin** and **Albumin,** whose concentration, in line with their function, is maintained at extremely low levels [46]. However, the abundance of Heme inside animal cells and tissues is such that it provides invading bacteria with a large potential reservoir of Fe. Several pathogenic bacteria species, including ***Bordetella bronchiseptica*** [47], ***B. pertussis*** [47], ***E. coli*** [24], ***Yersinia spp***. [48,49], ***N. meningitidis*** [43], ***N. gonorrhoeae*** [43] ***H. influenzae*** [44] ***V. cholerae*** [45], ***C. diphtheriae*** [50] and ***Shigella spp***. [51], use Heme group as a source of Fe.

IROMPs presence has been reported in ***Bacteroides fragilis***, a clinically important, anaerobic, non spore-forming Gram-negative pathogen [52,53,54,55]. Other studies in ***B. fragilis*** **have demonstrated that a more virulent strain growth, in presence of serum and plasma, is feasible.** Interestingly, growth inhibition in serum and plasma can be abolished by the addition of ferrous sulphate [56,57]. The presence of **IROMPs** in *B. vulgate* and *B. fragilis* has been corroborated [58,59]. *B. fragilis,* specifically, showed a strong relationship between the bacterial virulence, the expression of a 44 kDa protein and its ability to grow in serum. There was no evidence of production of iron chelants [58,59]. By the same token, in *H. influenzae,* an outer membrane protein of 43 kDa was reported, which plays a role in Heme uptake [60,61]. Further investigations revealed that the 44-kDa protein of *B. fragilis* could be involved in the passage of Heme groups through the bacterial envelope [61,62].

As mentioned above, there is considerable evidence that suggests that the ability to infect of a large number of human pathogens is directly related to their ability to obtain iron [62]. ***Neisseria*** **produces many new proteins on its cell surface when the microbe is grown in iron-restricted conditions**. These proteins are **referred as iron-regulated proteins (IRPs)**. The *Neisseria* IRPs have a Mr ranging from 36 to above 100 kDa [63,64,65].

The role of **IRPs** in improving the ability of the organism to survive within the host has not been fully determined. Simonson *et al*. [66] found a 36.5-kDa protein that complexed strongly to radioactive-labeled iron which could be involved in the transport of this nutrient. This study showed that *Neisseria* has a 70-kDa protein that binds iron specifically. The meningococcus eagerness to internalize iron produces the expression of an outer-membrane TfR [67]. This is a specific and highly saturable receptor. Correspondingly, t**he IRPs of *N. meningitidis* are expressed *in vivo,* inducing antibody production at detectable levels during child infection** [68].

Moreover, validation studies of *N. meningitidis* reported **IROMPs as a potential vaccine**, especially the Tbp2: a Tf-binding protein of the bacterial surface. This study found that **Tbp2** is expressed by all strains of *N. meningitidis*, and it is immunogenic in mice and humans. In both cases, Tbp2 presented antigenic heterogeneity. The native purification of the complex formed by the TfR and Tbp2 allowed evaluating the bactericidal effect against the homologous strain, but not against the heterologous tested strains [69].

In investigations realized with IROMPs immunization of *M. haemolytica* A1 in cattle, revealed that cattle vaccinated with outer membrane proteins supplemented with IROMPs mainly produce antibodies against the 70 and 77-kDa (IROMP) proteins. Linear regression analysis indicated a significant correlation between high antibody response against outer membrane proteins enriched with IROMPs and the resistance to infection. **Overall, anti-IROMPs, in association with antibodies from another surface antigen, raised the immunity against pathogenic *M. haemolytica*** [70].

Similarly, in serological studies in *E. coli*, the serum of healthy human adults and infants, as well as the serum of mice, rabbits and guinea pigs, was examined. It was shown that **there are antibodies that naturally react against two outer membrane iron-regulated proteins from *E. coli* strains O111 and O118**. It was determined that some individuals have antibodies reacting very strongly with the **IROMPs**, including the ferric-enterochelin receptor (81 kDa) as well as with and an outer membrane protein (**OmpA**). In human serum the antibodies were immunoglobulin G class [71].

Consequently, **it is clear that iron limitation induces the expression of IROMPs**, which, at the same time, are not expressed when there is enough iron in the growth medium. IROMPs have been purified to assess their protective potential in experimental models. **An antiserum was prepared from IROMP in rabbits and the immunity was passively transferred to mice**. Antibodies anti-IROMPs provided 60% of protection against *Salmonella enterica* serovar Typhi at a 9.6 LD_{50} dose. The hyperimmune serum of anti-IROMPs proved to be bactericidal in the presence of complement, while the bacterial destruction was not observed in the presence of pre-immune serum.

The IROMPs (480 LD_{50}) provided protection to 90% of the assayed mice. The levels of different isotypes of antibodies (**IgG, IgM and IgA) in serum and secretory antibodies (IgA)** in the mouse intestinal fluids correlated with protection. In conclusion, t**hese studies indicated that anti-IROMPs antibodies play an important role providing a systemic and mucosa protection** [72]. However, **IROMPs not only generate humoral immune response, but also potentially participate in co-regulating the cellular immune response**. Such is the case of the IROMPs from ***Salmonella enterica* serovar Typhi** that modulate the specific response of T cells. In this study, the delayed immunity and lymphocyte proliferation induced by IROMPs were measured. T cells phenotype and the cellular production of cytokines using lymphocytes isolated from spleen and **Peyer's patches** were also evaluated.

The mice immunization with IROMPs increased the delayed immunity response and significantly increased the proliferative response of specific proteins of the spleen lymphocytes and

Peyer's patches. Furthermore, cytokines analysis revealed that during **an initial period the production of IL-2 and IFN-γ** was increased by lymphoid cells obtained from spleen and Peyer's patches, **indicating a Th1 response, whereas the production of IL-4 was later increased; this process suggested a Th2 type response**. To summarize, the results of this study suggest the role of *S. typhi* IROMPs in the modulation of the cellular immune response at peripheral and mucosa level [73].

Table 1. MOLECULES USED BY SOME BACTERIA AS AN IRON SOURCE [74]

Source	Bacteria
Heme	*Corynebacterium diphtheriae, Haemophilus influenza, Helicobacter pylori, Shigella dysenteriae, Yersinia pestis, Neisseria meningitidis, Neisseria gonorrhoeae, Vibrio parahaemolyticus, pathogenic Escherichia coli, Prevotella nigrescens, Streptococcus pneumoniae*
Haptoglobins	*Neisseria meningitidis* and *pathogenic Escherichia coli*
Hemopexin	*Haemophilus influenzae*
Transferrin	*Neisseria meningitidis, Neisseria gonorrhoeae, Mycobacterium tuberculosis, Moraxella catarrhalis*
Lactoferrin	*Neisseria gonorrhoeæ, Neisseria meningitidis, Streptococcus pneumoniae, Helicobacter pylori*
Ferritin	*Neisseria meningitidis*

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Chapter 11

Regulation of iron-controlled gene expression in *Escherichia coli*

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Abstract

1. Fur and its DNA binding site

2. Regulation of *fur* expression

3. Positive regulation by Fur

4. Iron regulation by RyhB and Fur

5. References

Abstract

Iron is an essential metal for most living organisms. It is found within the active site of enzymes that participate in DNA synthesis, in respiration, in the tricarboxylic acid cycle, and in the synthesis of several metabolites [1]. Despite that iron is the most abundant metal on the Earth's crust, its bioavailability is almost null, due to its very low solubility in water in the presence of oxygen at neutral pH (see the Chapter Iron and its biological function by I. Peñalosa).

Bacteria have mechanisms for iron uptake and systems that finely control the intracellular concentration of this metal. Whereas Fe^{2+} is an essential cation, it is dangerous when in excess, since it participates in the generation of the highly reactive hydroxyl radical (OH·) by the Fenton reaction.

E. coli genes involved in iron metabolism are transcriptionally-regulated by the regulatory Fur protein, and by the non-coding RNA RyhB at the post-transcriptional level. RyhB transcription is negatively regulated by Fur-Fe repressor. Fur synthesis is regulated by four mechanisms:

(a) Fur-Fe transcription repression of *fur* gene,

(b) RyhB-mediated translation inhibition of $mRNA_{fur,}$

(c) Translation of $mRNA_{fur}$ coupled to *uof* translation, and

(d) iron-responsive decoding of *uof.*

The aim of this chapter is to review the most relevant aspects of the regulation of iron-controlled gene expression in *E. coli.*

Key Words: Fur, iron, transcription repression.

1. Fur and its DNA binding site

In 1981, Hanke obtained an *E. coli* mutant able to express constitutively the iron uptake systems. He designated the mutation conferring this phenotype as *fur*, for **f**erric **u**ptake **r**egulator [2]. Later, *fur* gene was mapped at 15.7 min on the *E. coli* genetic map [3], and it was demonstrated that **it codes for a 17 kDa protein which contains Zn^{2+}** [4] **and repress the transcription of both iron-uptake and siderophore-biosynthesis genes.**

According to the regulation model, coordination of a Fe^{2+} atom with each Fur monomer allows homodimer binding to a specific 19 bp DNA sequence named **"Fur box"** located at the promoter region of the regulated gene.

Fur box has been interpreted as a **palindromic sequ**ence composed by two 9-bp inverted repeats; three direct 6-bp repeats, the third one being imperfect; or two direct and one inverted repeats of the invariable GATAAT sequence (Figure 1a [5, 6]). Most Fur boxes are overlapped with -10 and -35 sequences of the target promoters (Figure 1b, [5]), so, **Fur binding does not permit that RNA polymerase gain access to promoter, i.e. repression by Fur is carried out by steric hindrance of the promoter.**

Cristal structure of *Pseudomonas aeruginosa* Fur protein is known [7]; this protein contains two domains: **N-terminal domain accounts for the ability of the protein to bind DNA,** and C**-terminal domain is implicated in dimerization.** Fur has two metal binding sites; the regulator site 1 is occupied by Fe^{2+} and is located in the C-terminus, whereas structural site 2 is in the N-terminus, connects both domains and is occupied by Zn^{2+} (Figure 1c).

2. Regulation of *fur* expression

Regulation of *fur* expression is complex and is not fully understood yet (Figure 2). Oxidative stress regulators **SoxRS and OxyR stimulate *fur* transcription from distinct promoters** [8]. The *fur* gene is contained in the ***fdlA-uof-fur* tricistronic operon** [9], which can be **transcribed from P_{fldA} promoter in response to the SoxRS-system mediated induction, which is activated by superoxide.**

Superoxide activates the SoxR transcription factor, which induces the expression of the transcriptional activator SoxS, which in turn increases transcription of genes whose products protects against oxidative damage [10], *fur* included. The *fur* gene can be transcribed also from anyone of two CRP-activated, Fur-repressed, σ^{70}-dependent promoters: $P_{fur(a)}$ or $P_{fur(b)}$ from which the transcriptional unit contains only *fur*. Finally, transcription from P_{fur1} promoter gives rise to the *uof-fur* transcript [Figure 2], and is positively regulated by SoxS and by the peroxide-activated transcriptional activator OxyR [8].

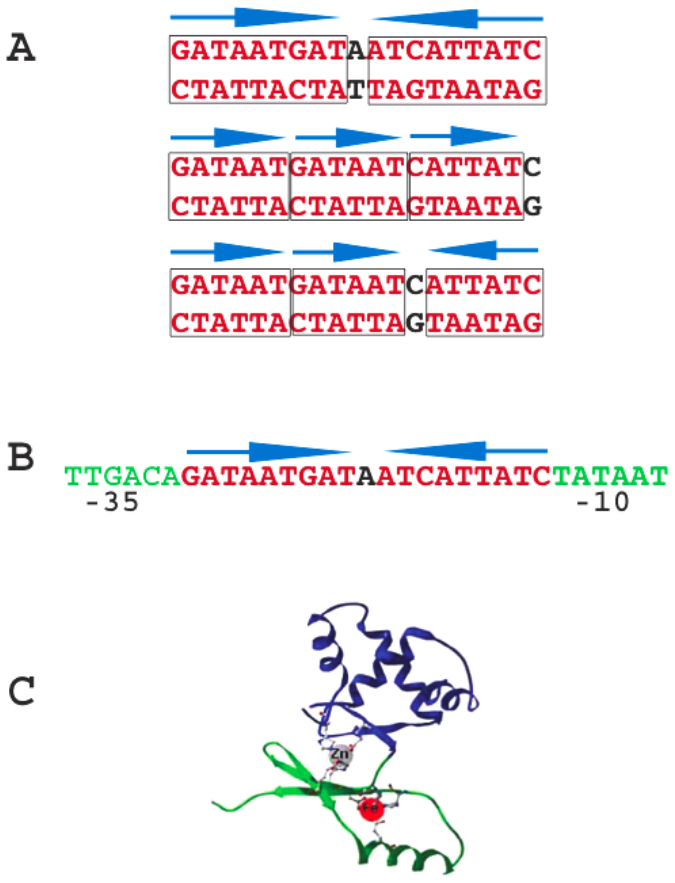
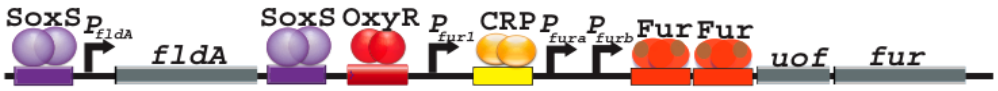


Figure 1. (A) NUCLEOTIDE SEQENCE OF Fur

(B) LOCALIZATION OF THE Fur-BINDING SITE (Fur BOX) WITHIN PROMOTER

(C) STRUCTURAL MODEL OF Fur MONOMER.

Expression of *fur* gene is post-transcriptionally regulated. It has been demonstrated that *fur* translation is coupled to translation of an upstream open reading frame named ***uof*** (***u***pstream ***o***f ***f***ur), translation of which is **downregulated by the non-coding RNA RyhB** (90 nucleotides) (Figure 2). RyhB and *uof-fur* mRNA pairing block off the ribosome-binding site, and later both RNAs are degraded by the degradosome (Figure 3) [11, 12]).



Promoter	Transcript	Activator	Repressor	Sigma
P_{fldA}	*fldA-uof-fur*	SoxS		?
P_{fura}	*fur*	CRP	Fur	70
P_{furb}	*fur*	CRP	Fur	70
P_{fur1}	*uof-fur*	SoxS OxyR		?

Figure 2. THE *fur* GENE CAN BE TRANSCRIBED FROM 4 PROMOTERS IN RESPONSE TO SoxS, OxyR OR CRP TRANSCRIPTIONAL REGULATORS. TWO PROMOTERS ARE NEGATIVELY REGULATED BY Fur AND ARE σ^{70}-DEPENDENT.

Figure 4 shows RyhB:$_{uof}$mRNA pairing. The mRNA is transcribed from P_{fur1} start at -183 nucleotide (in relation to +1, the first nucleotide of the *fur* start codon). **Shine-Dalgarno sequences** are located at -90 (*uof*) and -12 (*fur*); the last one being inaccessible to ribosome due to the formation of a stem and loop structure. By this reason, **translation of *fur* depends of *uof* translation,** which initiates at AUG codon (-79 to – 77 nucleotides). It has been demonstrated *in vitro* that terminus of *uof* translation is required for the start of *fur* translation, since substitution of the *uof* stop codons (+6 to +11 nucleotides) by sense codons significantly reduced *fur* translation [11].

An additional control in *fur* mRNA translation, termed iron-responsive decoding, is exerted by the UCA_6 and AGA_7 codons. MiaB protein is a Fe-S enzyme, whose activity is Fe-dependent, which participates in methylthiolation of adenosine 37 of almost all tRNAs that read codons starting with U [13]. Serine codon UCA_6 is decoded by the MiaB-dependent $tRNA_{IV}^{Ser}$, whereas AGA_7 codon is decoded by the rare $tRNA_4^{Arg}$. It has been described that AGA codons close to the initiation codon can lead to ribosome stalling and eventually release of mRNA [14, 15]. **Since iron limitation results in ~~a~~ lack of methylthiolation of A37, it has been suggested that UCA_6 AGA_7 codons in $_{uof\text{-}fur}$mRNA would limit translation of this mRNA in response to iron depletion and this could abate Fur synthesis in the absence of the co-repressor** [11].

3. Positive regulation by Fur

Some genes that codify for Fe-utilizing proteins are positively regulated by Fur. Among these are the ***sdhCDAB*** (**Succinate dehydrogenase**) and ***nuo*** (**NADH: Ubiquinone oxidoreductase, Complex 1**) operons, and the genes ***sodB*** (**Fe-Superoxide dismutase**), ***acn*** (**Aconitase**), ***fumA*** (**Fumarase**), and ***ftn*** (**Ferritin**). Fur-mediated **positive regulation is through repression of transcription of *ryhB*, whose product RyhB causes degradation of these mRNAs** (Figure 3). It has been reported that **RyhB regulates expression of 18 transcripts that encode 56 proteins that utilize iron** [16].

RyhB interacts with the RNA chaperone Hfq protein [17], which is necessary for its stability and activity [12]; **Hfq protects RyhB against RNAseE degradation** [18]. So, RyhB acts to diminish Fe^{2+} utilization when concentration of the metal is limiting. **RyhB role is to economize Fe, avoiding its use by non-essential proteins, whose synthesis is prevented by degradation of their mRNAs.** In this way, scarce iron can be used as cofactor by the essential enzymes requiring it.

Moreover, **RhyB expression increases 50% the intracellular concentration of free iron.** This iron increase depends only of just acquired iron and not of the iron already present inside the bacterial cell [19]. **This higher Fe concentration triggers Fur activation, and the Fur-Fe complex represses transcription of a subset of genes** (Box 1, [16]).

Temporal analysis of mRNAs degradation [***exbBD*** (**Fur regulated**), ***sodB*** (**RyhB regulated**)] by Northern blot, after *ryhB* expression induction from P_{BAD} with arabinose, allows proposing **a model for explaining the indirect effect of RyhB upon Fur-regulated genes.** The model postulates that **mRNAs paired with RyhB are degraded ~3 minutes after *ryhB* expression.** Without translation of mRNAs of Fe-utilizing proteins, intracellular concentration of iron increases, and this leads to Fe-Fur complex formation, which in turn repress transcription of several genes whose products take part in iron capture. About 7 minutes after RhyB expression, Fur-regulated mRNAs disappear (Figure 5, [16]).

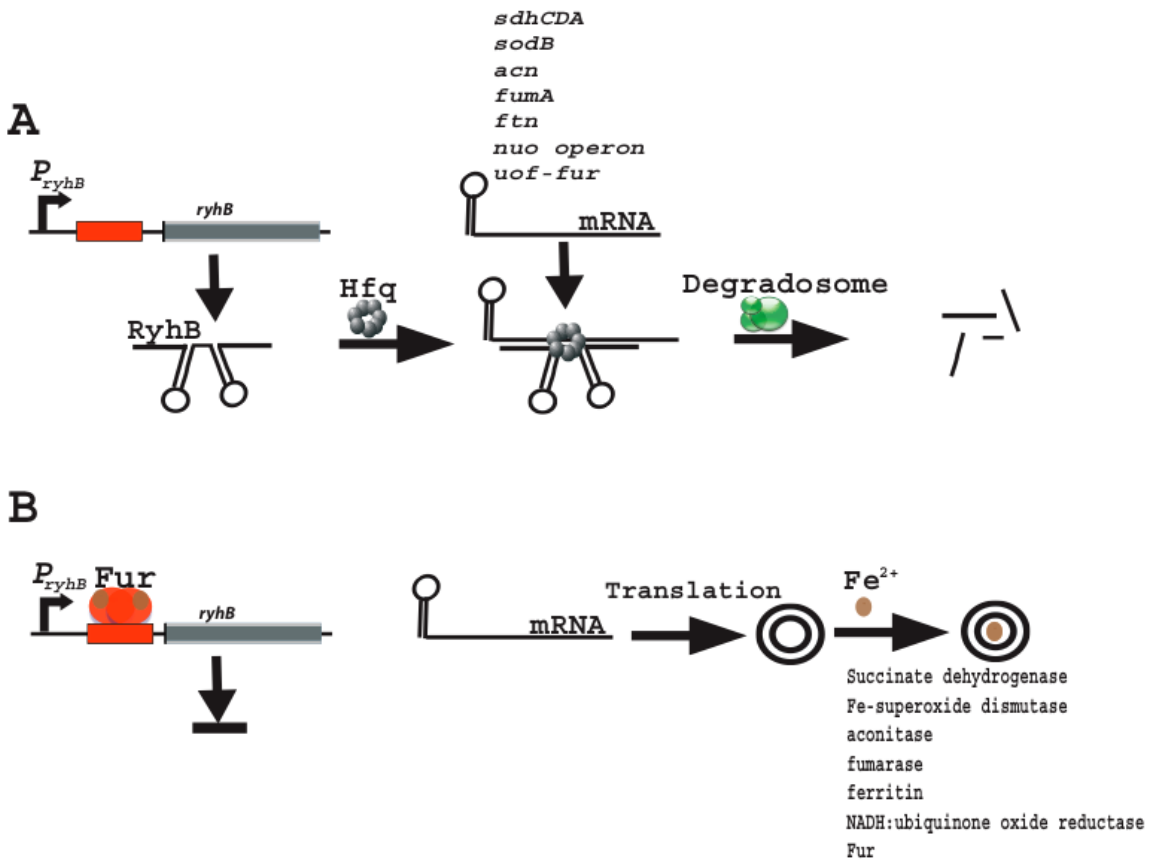


Figure 3. POSITIVE REGULATION BY Fur.

(A) When Fe^{2+} concentration is low, transcription of *ryhB* gene originates the small antisense-RNA RyhB, which is stabilized by the RNA chaperone Hfq. Pairing of RyhB with target mRNAs blocks their translation and permits that degradosome recognizes and degrades the RyhB:mRNA complex.

(B) When Fe^{2+} is sufficient, Fe-Fur complex repress *ryhB* gene transcription. In the absence of RyhB, mRNAs of the target genes are traduced and the proteins synthesized utilize Fe^{2+}.

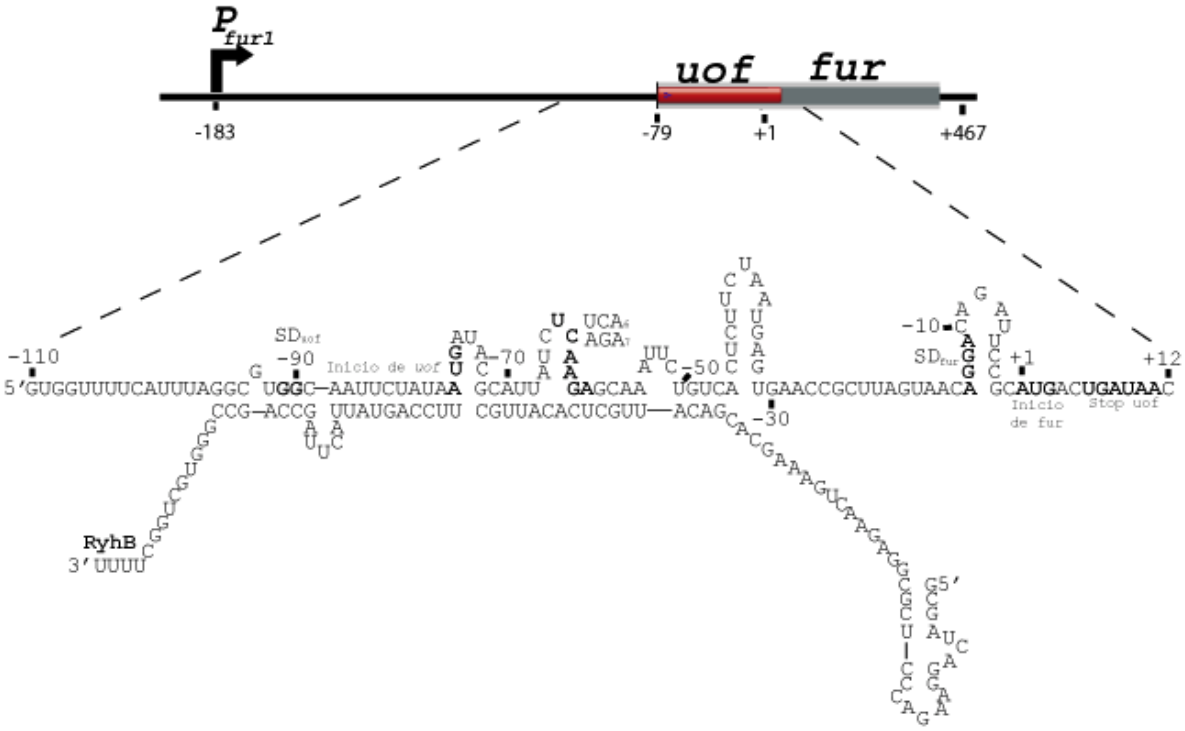


Figure 4. ORGANIZATION OF *uof-fur* TRANSCRIPT, AND MODEL OF THE STRUCTURE FORMED BY *uof* AND RyhB PAIRING.

Shine-Dalgarno sequences (SD_{uof} and SD_{fur}, with this last one being inaccessible to ribosomes due to the formation of a stem and loop structure); start codons (*uof*: -79 to -77; *fur*: +1 to +3); the two stop *uof* codons (+6 to +11) and UCA_6 and AGA_7 triplets, that participate in the decodification in response to iron, are shown [11].

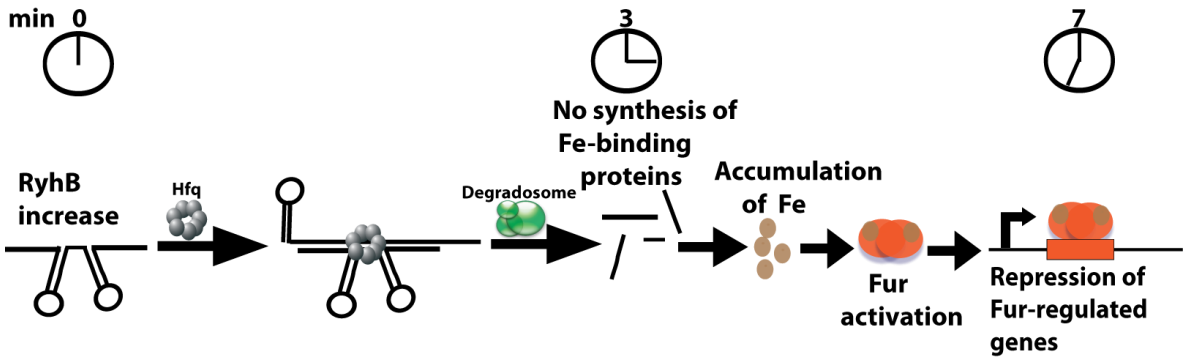


Figure 5. MODEL TO EXPLAIN THE INDIRECT EFFECT OF RyhB UPON Fur-REGULATED GENES. RyhB pairs with RNAs of target genes and causes its degradation (*sodB* mRNA disappears 3 min after RyhB expression).

In the absence of iron-utilizing proteins, the intracellular iron concentration rises and Fe-Fur complex is formed. Fe-Fur complex repress the transcription of another set of genes (*exbBD* mRNA disappears 7 minutes after RyhB induction, see Box 1).

Box 1. GENES REPRESSED BY Fur [16].

Gene	Description
fhuA	Outer membrane protein, ferrichrome and colicin M receptor
fhuC	Hydroxamate-dependent iron transport, ATP binding component,
fhuD	Hydroxamate-dependent iron transport, cytoplasmic membrane
fhuB	Hydroxamate-dependent iron transport, cytoplasmic membrane
ycdN	Iron permease (high affinity)
ycdB	Iron-dependent peroxidase
sufE	Stimulate SufS
sufS	Cistein desulfurase
sufD	Stimulate SufS, stabilize FhuF protein
sufC	Stimulate SufS
sufB	Stimulate SufS
sufA	Participate in iron-sulfur cluster assembly

exbD	Enterochelin transport, complex with TonB
exbB	Enterochelin transport, complex with TonB
bfr	Bacterioferritin
bfd	Ferredoxin associated to bacterioferritin
feoA	Ferrous iron transport, protein A
feoB	Ferrous iron transport, protein B
sodA	Mn-superoxide dismutase
fecE	Citrate-dependent iron transport, binds ATP
fecD	Citrate-dependent iron transport, membrane-bound protein
fecC	Citrate-dependent iron transport, cytosolic protein
fecB	Citrate-dependent iron transport, periplasmic protein
fecA	Citrate-dependent iron transport, outer membrane receptor
fecR	Regulation of ferric dicitrate transport
fecI	Probably regulates *fec* genes for dicitrate transport
fhuF	Ferric iron reduction in ferrioxamine B, binds to 2Fe2S cluster

4. Iron regulation by RyhB and Fur

Current model of iron regulation by RyhB and Fur is as follows: After iron entry to cell, it remains temporarily free before its incorporation into **essential** (**i.e. Ribonucleotide reductase**) and **non-essential** proteins (**i.e. Fe-Superoxide dismutase**) that utilize iron.

When intracellular iron concentration increases (by decreased synthesis of Fe utilizing proteins), active complex Fe-Fur is formed and represses transcription of genes whose products take part in iron intake. On the contrary, **when iron levels fall down, Fe-Fur complex dissociates and, in the absence of repression, transcription of *rhyB* and genes for iron intake is induced.** RyhB blocks translation of mRNAs coding for non-essential iron-utilizing proteins and this allows availability of just internalized iron for the essential proteins requiring it (Fig. 6, [20]).

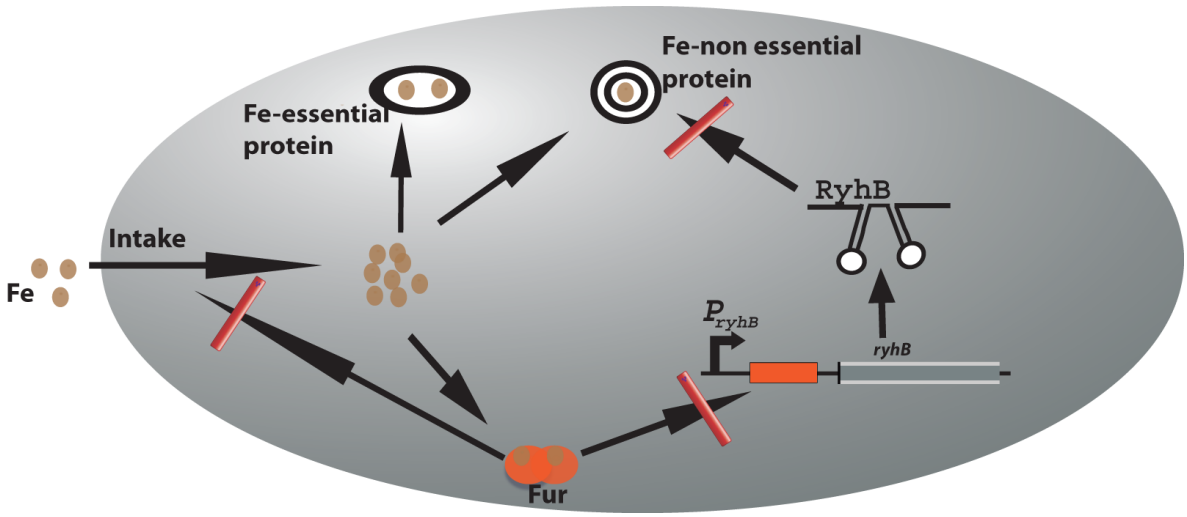


Figure 6. IRON REGULATION BY RyhB AND Fur.

After entering to cell, iron remains temporally free, until its incorporation into essential and non-essential iron-utilizing proteins.

Increased iron concentration activates Fur, which in turn represses transcription of *rhyB* and genes for iron intake.

When iron concentration falls down, Fur repressor is inactivated, transcription of *ryhB* and iron intake genes is induced. RyhB blocks synthesis of non-essential iron utilizing proteins, leaving the scarce iron freely available to being used by the essential proteins requiring it.

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Chapter 12

Pathogens as well as vampires need blood to survive

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Abstract

1. Iron uptake systems from hemoglobin or hemoproteins

2. Storage and regulation of heme group

3. Blood feeding parasites degrade hemoglobin

4. Conclusions

5. References

Abstract

Iron (Fe) is an essential nutrient for almost all living organisms. Its importance originates from the flexibility of iron redox potentials.

Parasites as well as other organisms need Fe for their survival inside their hosts. In mammals Fe homeostasis is strictly regulated. It is known that although there is plenty of Fe present in the body fluids of human and animals, the amount of free iron, which might be easily available to pathogens, is extremely small.

Host-iron availability has a crucial role in the host-pathogen relationship. As a general anti-microbial defense mechanism, mammals possess an elaborate iron-withholding system that effectively reduces the amount of iron accessible to pathogenic microorganisms. Most of the body´s Fe is found intracellular and the extracellular limited amount of Fe is tightly bound to proteins such as Transferrin and Lactoferrin. Therefore, invading pathogens use specialized and efficient mechanisms to acquire iron, and iron availability is often found to have an important role in virulence. Some potential invaders that have impaired iron-acquisition ability cause disease primarily in iron-loaded hosts.

Heme is a major iron source for bacteria and other pathogens, which are able to internalize heme as a whole molecule and to degrade it in the cytosol in order to retrieve iron. In most pathogens, heme uptake occurs by direct recognition of exogenous heme or host hemoproteins (Hemoglobin, Haptoglobin-Hemoglobin, and Hemopexin-Heme).

At last, some bacteria secrete proteins called hemophores that are able to acquire heme from diverse sources and to transport it to specific outer membrane receptors.

Key words: haptoglobin, hemoglobin, haptoglobin-hemoglobin complex, heme, hemin-receptor, hemopexin-heme complex, hemophore, hemoglobin-receptor.

1. Iron uptake systems trough Hemoglobin or Hemoproteins

Iron (Fe) is fundamental to the biology of almost all living organisms. Its importance originates from the flexibility of **redox potentials** available to iron by its varied interactions with coordinating ligands and from its capacity to undergo electron-transfer and acid-base reaction [1]. Iron is a co-factor of a variety of proteins that can be classified according to the coordination chemistry of the metal:

1) Hemoproteins, which function as O_2 carriers, O_2 activators, or electron transfer proteins;

2) Iron-sulfur cluster proteins, involved mainly in electron transference and

3) Non-heme, non-iron-sulfur, iron-containing proteins, including enzymes and proteins involved in iron transport and storage [1].

Iron is also an essential nutrient for nearly all microbes. To ensure Fe availability and to eliminate the toxicity of free Fe in addition to its accessibility for invading pathogens, mammals have evolved strictly regulated systems for iron homeostasis [2]. Mammalian cells divide much less frequently than microbes do, taking advantage of this vulnerability and using Fe starvation to infecting microbes as an important host defense strategy. In response to infection, mammals sequester Fe into iron-binding proteins (**Ferritin**, **Lactoferrin**, **Siderocalin**) [3] and locations less accessible to most invading microbes (cytoplasm of macrophages and hepatocytes) [4].

Bacteria are restricted in Fe whatever their biotope. When grown in acellular, aquatic, or soil media, bacteria mostly encounter insoluble Fe^{+3}. Even when they colonize multicellular organisms, whether they are **saprophytic** or **pathogenic** microbes, **they do not find freely available iron** [5]. Under conditions of Fe starvation, microorganisms must use all of the Fe sources found in their environment [5]. The majority of Fe in the body is stored intracellularly in proteins such as **Ferritin** and **Hemoglobin (Hb)**. The iron component of Hb, Heme, can be utilized as a source of essential iron by many microorganisms.

However, Heme is cytotoxic and is sequestered by host **hemoproteins** such as **Hemopexin (Hx)** [6]. To overcome the limited access to Heme, pathogenic bacteria commonly secretes exotoxins such as hemolysins [7, 8], **cytolysins** [9], and **proteases** [10] that can lyse cells and release the Heme bound to host carrier proteins such as Hb that reside in erythrocytes. For example, the ***Escherichia coli*** Hb protease (**Hbp**) was found to be involved in the symbiosis of pathogenic bacteria *E. coli* and ***Bacteroides fragilis*** [11]. Hbp is part of **SPATE** family (serine protease autotransporters of *Enterobacterriaceae*) and it degrades Hb, releasing Heme to both bacterial species [11].

Three major Heme acquisition systems have been identified in Gram-negative bacteria [12]. The well characterized system involves direct binding of Heme or heme proteins (**Hx-Heme, Haptoglobin (Hp)-Hemoglobin**) to specific outer membrane receptors on the bacterial surface and relies on cell surface proteins that bind iron or heme-containing molecules (named **"Direct Heme Uptake System"**) [12] (Figure 1). The heme is then transported into the cell via **ATP-binding-cassette (ABC)** transporters [12]. In this category are: *hemR-hemSTUV* in ***Yersinia enterocolitica*** [13], ***hmuRSTUV*** in *Y. pestis* [14], ***shuASTUV*** in ***Shigela dysenteriae*** [15], and ***phuRSTUVW*** in ***Pseudomonas aeuroginosa*** [16] (Figure 1).

It has been demonstrated that the **Direct Heme Uptake System is capable of transporting intact porphyrin from Hemoglobin and the Hp-Hb complex.** For example, two receptors that utilize Heme as an iron source have been identified in ***Neisseria meningitides***: **HpuA/HpuB** [17] and **HmbR** [18]. **HpuA** is a 36 kDa lipoprotein and **HpuB** is an 85 kDa, **Ton B-dependent,** outer membrane receptor.

The HpuA/HpuB receptor is a two-component receptor analogous to TbpB/TbpA (the bacterial receptor for Transferrin). It binds Hb, Hp-Hb, and apo-Hp (without iron). **HmbR** is an 85 kDa outer membrane receptor that **shares amino acid similarities with Ton B**-dependent receptor. Unlike HpuA/HpuB, **HmbR is not a two-component system and is specific only to Hb** [19].

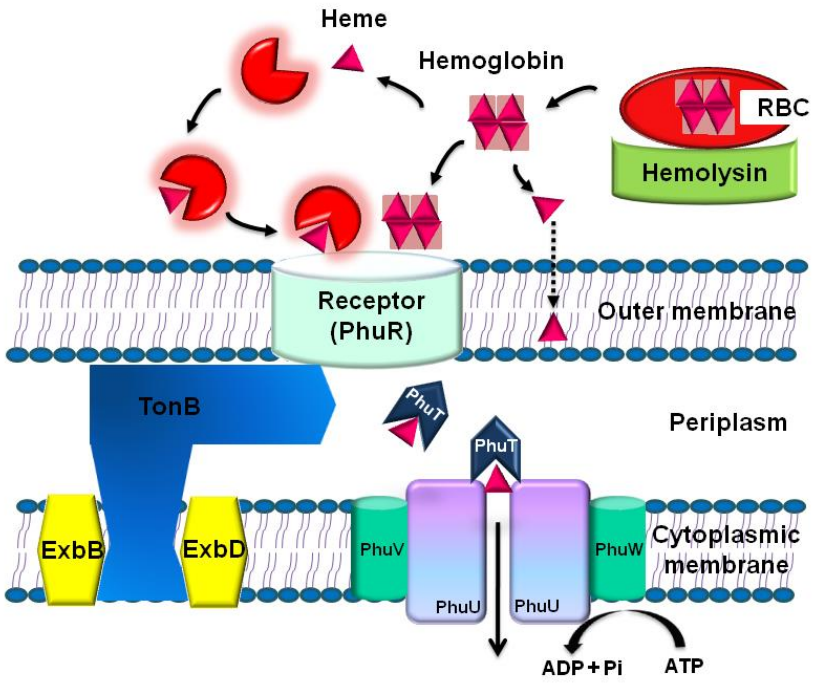

Figure 1. SCHEMATIC REPRESENTATION OF HEME-IRON BINDING RECEPTOR SYSTEMS IN GRAM-NEGATIVE BACTERIA

The second Heme uptake system is comprised of an outer membrane receptor, an **ABC transporter** and **extracellular hemophores, which** are specialized bacterial proteins that extract iron from Heme [12] (Figure 2). Hemophores have been identified in many Gram-negative bacteria.

One type of hemophore is **HxuA** from ***Haemophilus influenzae*** that binds Hx [20]. A second hemophore acquisition system **(HAS)** has been identified in ***P. aeuroginosa, P. fluorescens, Serratia marscescens, Y. enterocolitica* and *Y. pestis*** [21, 22]. The hemophore Has A form a family of highly conserved proteins, HasA is secreted by the Type I secretion pathway **(TISS)** into the bacterial environment by an ABC transporter composed of a cytoplasmic membrane ABC protein **HasD,** a second inner membrane protein **HasE,** and the outer membrane component, **HasF** [23, 24] (Figure 2).

Once in the medium, **HasA is able to capture Heme from Hb of diverse species** (human, bovine, leghemoglobin, etc). It captures Heme regardless of the Heme redox status (Fe^{3+} or Fe^{2+}). **HasA is also able to capture Heme from Hx and Myoglobin** [24]. **This wide range of substrates suggests a passive transfer of Heme from the heme carrier protein to the hemophore due to its higher affinity, rather than a protein-protein interaction between the hemophore and the heme carrier protein** [25].

HasA binds to its specific receptor HasR with high affinity [25] (Figure 2). HasR can also recognize a wide variety of substrates including free Heme, Hb, Hp-Hb, and Heme-Albumin [25]. **Heme transfer from Has to Has R is driven by protein-protein interactions.** The presence of the hemophore greatly enhances the efficiency of Heme uptake by HasR [25].

HasR-mediated heme entry into the cell is a **TonB complex dependent** [26] (Figure 2). Hemophore recycling is energy-dependent. Thus, the **Ton B complex acts at both the Heme entry step and as apo-Has recycling in the extracellular medium.** It is thus likely that HasA mediated Heme entry *per se* requires more energy than free Heme entry, and that both steps (Heme entry, and HasA ejection) are tightly linked [27]. Once the Heme has been transported to the cytoplasm it is degraded [28].

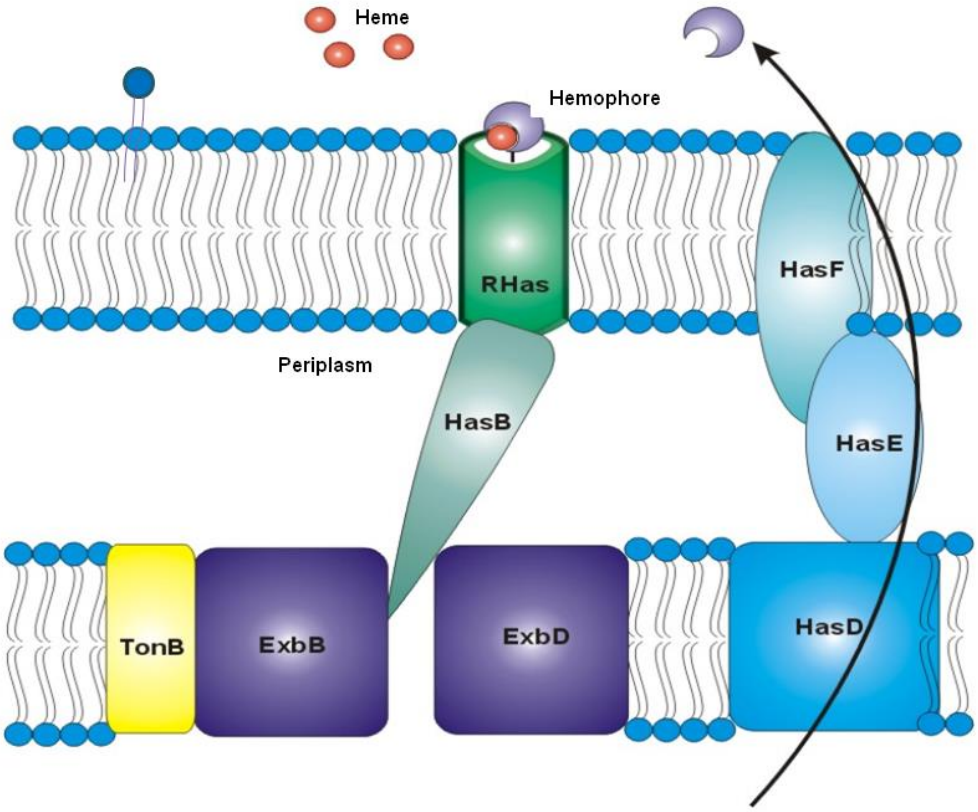

Figure 2. SCHEMATIC REPRESENTATION OF HEMOPHORE ACQUIRING SYSTEM

A third system is the bipartite Hb receptor HpuAB in *N. meningitidis* [29]. HpuAB is composed of a Ton B-dependent outer membrane receptor HpuB and an accessory outer membrane lipoprotein HpuA [30], which is analogues to the bipartite Transferrin receptor Tbp1 and Tbp2 [31].

Unlike that of Gram-negative bacteria, the envelope of **Gram-positive bacteria have a thick cell wall** composed of **murein sacculus** and the attached **polysaccharides, teichoic acids, and cell wall proteins** [32]. Studies of bacterial Heme uptake systems have been mainly focusing on Gram-negative bacteria. However, some studies done in Gram-positive microorganisms such as ***Corynebacterium diphtheriae*** have found that a **Heme T-like lipoprotein, HmuT serves as Heme receptor** [33]. The ABC transporters are required for Heme uptake not only in *C. diphteriae* [33] but also in ***Streptoccocus pyogenes***, and ***S. aureus*.**

Cell surface binding proteins (named **Shr and Shp**) and the ABC transporter lipoprotein (named **HtsA**) in human pathogen group A *Streptococcus* have been recently identified [34] (Figure 3). The *Gram*-positive bacterium ***S. aureus* has developed a Heme-acquisition system encoded by an *isd* (iron-regulated surface determinants) gene cluster to acquire Heme-bound iron during infection, via the secretion of hemolysins** [35].

The Heme uptake systems of Gram-positive bacteria consist of a cell surface protein and an ABC transporter [36]. For example, the Heme uptake system in *S. aureus* consists of the cell surface proteins **IsdA, IsdB,** and **IsdH** responsible for the binding of Hx-Heme, Hb, and Hp-Heme, respectively, covalently attached to the cell wall [36]. The inner membrane transporter, **IsdDEF,** transports the Heme across the plasma membrane into the bacterial cytoplasm where it is degraded to **Biliverdin, carbon monoxide,** and free iron by the **Heme Mono-oxygenase IsdG** and **IsdI** [37].

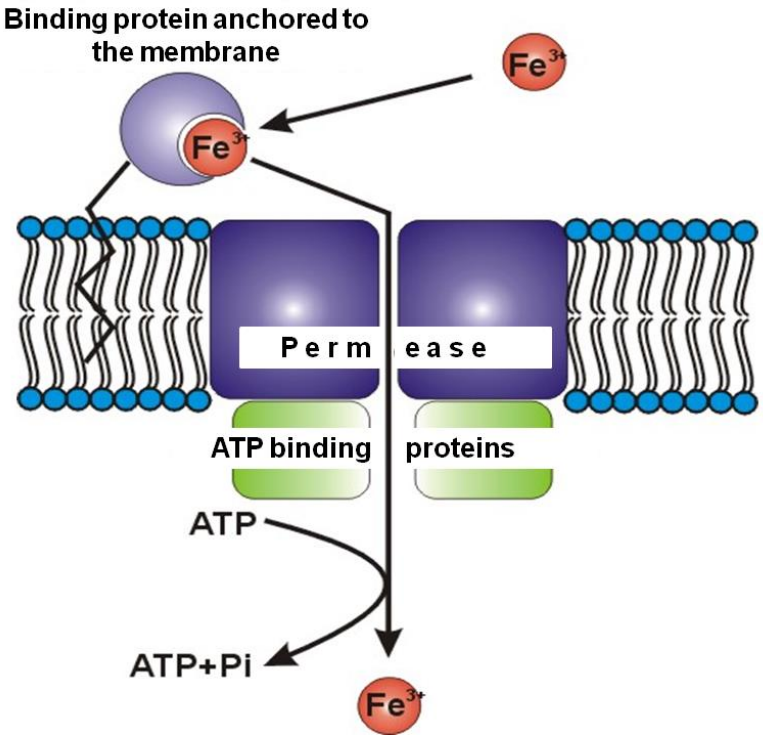

Figure 3. SCHEMATIC REPRESENTATION OF HEME-IRON BINDING RECEPTOR, USING PERMEASES FOR ITS TRANSPORTING IN GRAM-POSITIVE BACTERIA

Heme transport across the outer membrane is an energy-requiring process. The energy for transport of Heme into periplasmic space in most Gram-negative organisms is provided by a cytoplasmic membrane protein **Ton B in association with two helper proteins, ExbB** and **ExbD**, which form a complex in a molar ratio of 1:7:2, respectively [38]. This is an active process driven by the **proton motive force** generated from cytoplasmic membrane TonB-homologues, this process is widespread in bacteria where they may have similar functions in Heme uptake systems [39].

Once acrossed the outer membrane, **Heme is internalized into periplasm and is then bound by a Heme-specific periplasm transport protein (HTP)**, which mediates Heme transfer to the cytoplasm through **ATP binding cassette (ABC) transporter** [40]. The ABC transporter mediates active translocation of a variety of molecules across cell membrane in both Gram-positive and Gram-negative pathogens. **When**

associated with heme specific HTP, it forms a complete Heme-uptake Permease. The first Heme permease was identified in *Y. enterocolitica*, composed of one periplasmic Heme-binding protein **(HemT)**, one transmembrane protein **(HemU)**, and one ATPase protein **(HemV)** [41].

Once internalized into the cytoplasm, Heme is rapidly catabolized by heme oxygenase (HOs), **which opens the heme porphyrin ring.** HOs, first identified in mammals, is primarily involved in protecting cells against the toxic effect of Heme, whereas **bacteria utilize HOs to access Heme as a nutrient iron source** [42, 43]. HO is ubiquitous in nature, and function to catalyze the oxidation of Heme into Biliverdin, carbon monoxide, and free iron. However, **before Heme is delivered to HOs, it may be sequestered by an inner membrane protein, because the free Heme is toxic to cells.**

The mechanism of **Heme oxidation by HO** has been proposed as follows: the reaction starts with the **formation of the ferric Heme-HO complex** with histidine as an iron ligand. **Ferric iron is then reduced** to a ferrous iron by the electron donated from **NADPH-Cytochrome P450 Reductase**. Next, **oxygen binds to the complex forming a metastable oxy-form.** The iron-bound oxygen is **converted to hydroperoxide intermediate (Fe^{3+}-OOH),** by receiving another electron from the reductase and a proton from the distal water pocket. Lastly, **the terminal oxygen of Fe^{3+}-OOH attacks the α-meso-Hydroxyheme, which is converted to the ferric Verdoheme in the presence of oxygen.** The mechanism of the verdoheme degradation to ferric biliverdin is not well understood.

2. Storage and regulation of the Heme group

Heme byosynthesis is strictly regulated. Free cytoplasmic Heme binds to **HemA** protein, which catalyzes the first step of Heme byosynthesis and promotes its proteolytic degradation [44]. **Most organisms have free Heme pool and known Heme storage proteins.** Iron is stored in Ferritins, found in most living organisms, and in **Heme-containing Bacterioferritins**, **found only in prokaryotes.** Ferritins and bacterioferritins are composed of 24 identical subunits. In addition, Bacterioferritins have up to 12 Heme groups of unknown function. Several bacterial Ferritins have been crystallized and all of them have similar spherical structure with a central iron storage cavity.

Ferritins and Bacterioferritins use oxygen and peroxide, as the electron acceptor, respectively, bind soluble Fe^{2+}, and catalyze its oxidation to diferric compounds. The insoluble ferric ions are deposited in the central cavity, forming the **ferrihydrite core** of about 5000 iron ions [45]. On the other hand, in bacteria that require exogenous Heme, the existence of **Heme storage proteins** has been suspected. Several homologous proteins including **HemS, ShuS**, and **HmuS** are encoded by genes located in operons involved in Heme uptake, and were first considered as Heme oxygenases, they might instead be Heme storage proteins [46].

Iron is an essential nutrient for bacteria; however, overload iron could be harmful due to its toxic properties. Therefore, **expression of iron/Heme uptake proteins during different periods of time of infection is regulated in response to iron level in the environment.** In many bacterial pathogens, such as *E. coli* [47], and *Salmonella typhi* [48] iron/Heme uptake regulation is **negatively controlled by the global determinant ferric-uptake regulator protein (Fur)**.

Fur is an homodimer consisting of 17-kDa subunits, it is divided in to two domains: N-terminal domain, which functions in DNA binding, and the C-terminal domain, which is rich in His residues and is responsible for divalent iron binding and dimerization. Fur regulon depending on the bacteria, **50 to 100 genes are negatively regulated by Fur in iron-rich medium** [49]. These genes are involved in diverse functions including iron and Heme acquisition, virulence factor production, and acid resistance [49].

Another regulatory mechanism involves sigma factors belonging to the family of extra-cytoplasmic function **(ECF)** sigma factors. These sigma factors have diverged significantly in some ways from the main sigma 70 factors. ECF sigmas involved in iron acquisition, also called **iron-starvation sigmas are highly conserved and form a distinct branch in the ECF sigma subfamily** [50]. They are widespread in Gram-negative bacteria and may represent the major mechanism of positive regulation for iron/Heme uptake genes. They are also found in Gram-positive bacteria but they have not been extensively studied.

3. Blood feeding parasites degrade Hemoglobin

Unlike pathogenic bacteria, **little is known about iron uptake and its intracellular metabolism in unicellular eukaryotic pathogens.** A wide variety of unicellular (yeasts) and filamentous fungi have developed sophisticated mechanisms of iron uptake. Among these organisms ***Candida albicans*** is an important opportunistic human pathogen**. During systemic infection, possible iron sources for *C. albicans* are Heme and Transferrin** [51].

The first step in Hb utilization by *C. albicans* in vivo is probably via binding to erythrocytes. *C. albicans* **hyphae are able to rosette erythrocytes via complement-receptor-like molecules** [52]. This pathogen has shown that **produces an hemolytic factor probably a mannoprotein attached to the fungal cell surface and is able to use hemolytic factors as an iron source** [53].

Heme uptake is probably a widespread mechanism of Fe acquisition in pathogenic fungi, but it is still poorly documented. Moreover, **specific surface Hemoglobin-receptors are involved in Hb uptake.** Weissman and Kornitzer [54] first identified the Hb-receptor gene family, two of which **(*RBT5* and *RBT5*/*PGA10*)** have been characterized in vitro as possessing Hb-binding properties**. Additionally, it has been shown that Hb is bound to Rbt5 and internalized via an endocytic pathway by yeast cells** [55]. Once Hb is internalized inside vacuoles, it must be **hydrolyzed or denatured by vacuolar proteases and/or**

acidic vacuolar pH to release Heme [55]. ***C. albicans* possess a Heme oxygenase Hmx1**, because the heme group requires the porphyrin ring to be opened, **Hmx1 is regulated by Hb and iron** [56]. The role of heme uptake in virulence and the mechanism of Heme import remain poorly understood.

Histoplasma capsulatum, a dimorphic fungus that causes respiratory illness in immunocompromised individuals, resides in macrophages during mammalian infection. It was shown that ***H. capsulatum* is able to use either hemin or Hb as an iron source and possesses a cell-surface protein for binding hemin in an iron-dependent fashion** [57].

Paragonimus westermani is a typical digenetic parasite, which can cause a paragonimiasis in humans. Host tissues blood cells are important sources of nutrients for its development, growth, and reproduction. This parasite has a 47 kDa **Cysteine hemoglobinase** that is able to degrade human Hb at acidic pH (from 3.5 to 5.5), the hemoglobinase is localized in the epithelium of the adult worm intestine implying that protein may act as a digestive enzyme for acquisition of nutrient from host Hb [58].

Other blood-consuming parasites depend on Hb catabolism for survival, and several putative Hb degrading enzymes have been already characterized in protozoa (***Plasmodium falciparum*** [59, 60], ***Plasmodium vivax*** [61], and ***Trichomonas vaginalis*** [62, 63] and in helminths (***Ancylostoma caninum*** [64], ***Strongyloides stercoralis*** [65], ***Haemonchus contortus*** [66], *and* ***Schistosoma mansoni*** [67]). These proteolytic enzymes have been shown to belong to either **cysteinyl** or **aspartyl** families of peptidases [68].

Blood feeding parasites employ a battery of proteolytic enzymes to digest the contents of their bloodmeal. **Initially most of them use aspartic proteases** to make initial or early cleaves in ingested host Hb. Based on this evidence, it is proposed that aspartic proteases catalyse a defined scission, thereby exposing other sites to digestion by a cascade of proteases (mainly aspartic and cysteine proteases) [68].

4. Conclusions

The Hb contained in blood erythrocytes is a very important source of iron and aminoacids for pathogen microorganisms, since bacteria to eukaytotic pathogens such as worms, protozoa, yeast and fungus. All these organisms have developed the adequate mechanisms to obtain Hb or its byproducts (Heme and globin) from the host. These mechanisms go since the production of surface receptors that recognize Hb, Hp-Hb, and Hx-Heme complexes.

Other mechanism involved in Hb uptake is the secretion of hemophores that bind Hb or the complex Hp-Hb, and the complex Hx-Heme and then the hemophore-protein complex is recognized by surface receptor and internalized inside the microorganism. The production of proteases that are able to degrade Hb is another Hb uptake mechanism that degrades the globin in amino acids releasing the Heme group, and then microorganism's Heme oxigenases open the Heme ring releasing the iron that is then stored in Ferritin-like

proteins. All these mechanisms are regulated by external concentrations of Hb or iron. The study of the mechanisms of uptake and utilization of iron and Hb by pathogen organisms is necessary to comprehend and develop new strategies such as antibodies against the surface receptors, or drugs that block the entrance of Hb and iron that are important to the develop and growth of pathogenic organisms.

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Chapter 13

Transferrin delivers iron not only to all body cells, but also to pathogenic microorganisms

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Abstract

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Abstract

Iron is a crucial element for life on Earth; the majority of organisms need this element to live. Normally iron is found in an insoluble state, thus, living beings have developed several strategies to obtain this metal from the environment.

After the humans obtain iron from the diet, this element is stored, bound to proteins, or exported to the serum. In the serum, iron is bound to Transferrin (Tf), which transports iron to every cell in the body. Each cell possesses a specific Tf receptor (TfR) in the membrane surface, which binds and endocytoses holoTf to obtain iron.

Parasitic organisms must obtain iron from their hosts, in which, due to a severe iron regulation, they face an extremely low concentration of this metal. Pathogenic organisms obtain this nutrient by mechanisms grouped in five main categories:

(1) By binding of iron-complexed proteins from its host through specific receptors;

(2) By the production of siderophores that compete with host proteins for this metal;

(3) By the secretion of specific proteases capable of cleaving host iron-binding proteins;

(4) By reduction of ferric to ferrous iron, a more soluble form, by means of ferrireductases; and

(5) By endocytosis of whole host iron-binding molecules.

All mechanisms are complex; one or several proteins participate, often conferring the specificity to the host. Since iron plays an essential role in the survival of microorganisms, the study of the systems employed to obtain this micronutrient may serve in the development of vaccines and therapeutic agents.

Key words: bacteria, iron, parasites, transferrin, transferrin binding proteins, transferrin receptors

Introduction

Iron (Fe) is an important micronutrient for the majority of organisms, except for lactic bacteria, which employ manganese and cobalt instead of iron [1].

In biology, iron exists in either the ferrous reduced form (Fe^{2+}) or the ferric form (Fe^{3+}). Iron is incorporated into proteins in the **catalytic centre** or as a part of the **electron transporting machinery** [2, 3]. It is indispensable for various activities such as photosynthesis, respiration, oxygen transportation, gene regulation, DNA biosynthesis and other processes involved in cellular growth.

Although iron is abundant in nature, **it is not normally found free in the biologically-relevant ferrous form, because** the **ferrous ion, via the Fenton reaction, produces reactive oxygen species which damage all class of biomolecules** [2, 3]. On the other hand, under aerobic conditions, the ferric ion is unstable. To avoid toxicity**, ferrous as well as ferric iron is complexed to a variety of proteins, for example, Transferrin, Lactoferrin, Hemoglobin, Cytochromes, Ferritin, etc. As a consequence,** free iron concentration in fluids is very low, reaching levels **as low as 10^{-18} M** [4, 5].

Transferrin (Tf) binds 1% of the total iron in the human body. In neutral pH conditions, this protein is maintained in the serum at a saturation of approximately 33%. Therefore, **it is able to effectively capture free ferric iron. Lactoferrin (Lf) is similar to Tf in both structure and the function of binding iron.** Lf mainly captures iron in mucosae and in the acidic conditions at inflammation sites. **Hemoglobin (Hb) inside mammalian erythrocytes binds iron in the heme group, in the ferrous form.**

For a pathogen establishes itself within its host, it must successfully colonize tissues. In addition, the intruder depends on both the availability of nutrients and the ability to obtain them. **The evolutionary success of a parasitic pathogen resides in their extraordinary capacity to adapt to the environmental rigors, including the lacking of free iron imposed by its host** [6].

The process of iron acquisition, even in rudimentary organisms, is extremely complex and it manages to meet the biological requirements of **necessity, insolubility, and toxicity.**

2. Prokaryotes

2.1. Gram-negative bacteria

Several bacterial genera can use iron-charged Tf (holoTf) from the host to obtain Fe. Two main strategies have been described for iron acquisition in Gram-negative bacteria:

(1) by secretion of siderophores that remove the iron from holoTf and after that the iron-charged siderophore is recognized by a receptor in the outer membrane, **or**

(2) by specific receptors to holoTf in the outer membrane [3, 7].

Some bacteria can use both mechanisms to acquire iron and are a clear example of their adaptation to the host environment. The major difference between the two mechanisms is that **ferric siderophores are internalized into the bacterial cell as intact molecules, whereas Fe complexed to Tf must be released before being transported inside the cell.** Reduction of Fe^{3+} to Fe^{2+} occurs in the cytoplasm or on the cytoplasmic side of inner membrane, as an essential step for iron incorporation into enzymes and storage proteins.

The required proteins for the siderophore biosynthesis are regulated by the repressor protein Ferric **u**ptake **r**egulator **(Fur).** ***Aeromonas salmonicida***, the causal agent of the salmon furunculosis, possesses a system that acquires iron from holoTf through the **catecholate siderophore** [8]. This bacterium presents a **receptor to siderophore in the external membrane,** encoded by *asbD, asbG,* and *asbC,* which are Fur regulated genes. Iron from host proteins, like holoTf, is captured by this siderophore, which has more affinity for iron than Tf. **Additionally, there is a report of extracellular enzymes able to reduce ferric to ferrous iron** [9]**, but none of them have been cloned or purified until now.**

Neisseria **and** ***Haemophilus*** are the most studied Gram-negative bacteria concerning iron-acquisition systems [10, 11, 12]. ***N. gonorrhoeae*** and ***N. meningitidis*** are human pathogens that cause gonorrhoea and meningitis, respectively. These bacteria **have evolved** strategies to capture iron in limiting conditions. One such strategy is **high affinity reception** by the **Tf binding proteins A and B (TbpA, TbpB), Lf binding proteins A and B (LbpA, LbpB)** and **the Haptoglobin (Hp)-Hb receptor HpuAB.** Each one of these receptors consists of **a lipoproteic subunit and a TonB-dependent porin** [13].

The bacterial outer membrane is a permeable barrier that offers protection from toxins, degradative enzymes, detergents, etc. The presence of **β-barrel proteins (named porins) permits** the passive diffusion of small solutes, whose molecular mass is lower than 600 Da [3, 14]. **Tf, an** 80 kDa protein, **cannot pass through the outer membrane.** Hence, bacteria need a receptor to bind and take up the iron from this protein.

Fe^{3+} is released from holoTf and transported through the outer membrane by a complex of Tf binding proteins: TbpA/TbpB, by an energy-dependent mechanism. This membrane lacks both a predetermined ion gradient and ATP needed to provide the energy for transport. **ATP is provided by TonB and two other associated proteins (ExbB and ExbD) found in the cytoplasmic membrane (Figure 1).**

Once Fe^{3+} is in the **periplasm, it is transported into the cytosol by the FbpABC transporter.** This complex is comprised of an iron-binding protein in the periplasm: **FbpA,** and an **ABC transporter,** composed of a permease on the membrane (**FbpB**) and an ATP-binding protein (**FbpC**) [3, 6, 10, 14].

By studies of genetic complementation and sequence analysis, a gene was found that encodes for the protein **FbpA.** This gene belongs to the ***fbpABC* operon,** whose products behave like transporters with a site for ATP binding. **These unidirectional transport systems are ubiquitous in bacteria. Not only do they sequester limiting nutrients, but they also transport them through the periplasm to the cell interior** [6].

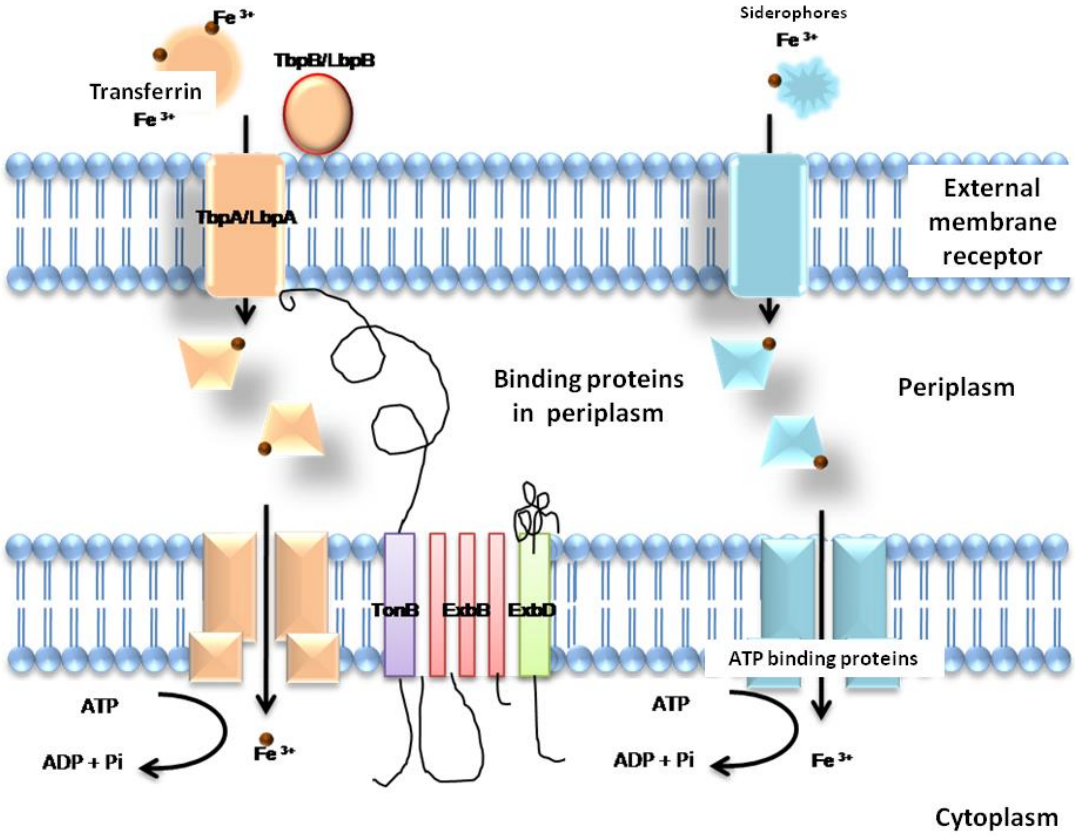

Figure 1. SCHEMATIC REPRESENTATION OF IRON CAPTURE BY TRANSFERRIN IN GRAM-NEGATIVE BACTERIA.

This system requires a receptor in external membrane, a binding protein in periplasm (PBP), and an ABC transporter in internal membrane. The transportation by the internal membrane receptor requires the participation of the TonB system, and the proteins ExbB and ExbD.

TonB contains both transmembranal and periplasmic domains. Its C-terminus interacts with the receptor in membrane. Moreover, **ExbB** is a cytoplasmic protein with three transmembranal regions, while **ExbD** is very similar to TonB. Both **ExbB and ExbD connect the TonB activity with the gradient of protons in the cytoplasmic membrane** [3].

TbpA is an integral membrane protein with large loops in the surface. This protein **forms a complex with holoTf and separates the regions around the iron-binding site**, thus releasing the metal [3, 14, 15, 16].

TbpB is a 65 kDa protein that is anchored in the outer membrane by the lipidic N-terminal region [15]. This region, at the same time, acts as an initial binding site for iron-saturated Tf and facilitates its subsequent interaction with TbpA [16].

When the iron arrives to the periplasm, it binds to the FbpA protein (37 kDa), a member of the Tf super family, with 20% identity to the Tf sequence. It is referred to as the **bacterial Tf,** due to the **functional and structural resemblance with mammalian Tf** [3, 17, 18, 19, 20]. **FbpA contains a unique site with a similar assembly to one functional lobe of the mammalian Tf.** Fe binds to tyrosines occupied with the exogenous anion, bicarbonate for Tf, and phosphate and sulphate for FbpA [11]. The role of the synergic anion *in vivo* remains an important biological question, whose answer may shed light on the mechanisms of iron assimilation in bacteria.

FbpA contains two domains with two lobes with an alternate α-helix/β-fold and connected by two anti-parallel β-strains. **The ferric ion binds to a region between the two domains.** The free and the iron-saturated structures were determined in ***Neisseria spp., Haemophilus influenzae,*** and other pathogenic bacteria [20, 21, 22, 23, 24, 25]. Like in other TonB systems, FbpA could have an important role in transporting Fe through the internal membrane, but its contribution in the transport and release of iron is not very clear [25].

The same proteins TbpA and TbpB involved in Fe acquisition were also reported in the swine pathogenic bacteria ***Actinobacillus pleuropneumoniae*** **and** ***Actinobacillus suis.*** These proteins **are expressed in iron-scarce conditions and are specific to pig holoTf. Therefore, they are considered as potential candidates for vaccine development** [15, 19, 24, 25, 26, 27, 28, 29, 30].

Klebsiella pneumoniae is a human pathogen involved in multi-drug resistant intra-hospital infections. This is another bacterium capable of utilizing holoTf as a sole iron source. According to a study where this bacterium was incubated in the presence of **holoTf and norepinephrine,** this stress hormone enhanced the bacterial growth by 500 times [31]. The same phenomenon was seen in both ***Bordetella bronchiseptica*** and ***Bordetella pertussis***, which indicates that this iron-acquisition mechanism is also present in these microorganisms; the former bacterium is the causal agent of the atrophic rhinitis in pigs, while the latter causes the whooping cough in children.

Genes homologous to those encoding for TbpA/TbpB receptor are absent in the genome of ***Klebsiella*** **and** ***Bordetella,*** from which only two proteins of low molecular mass are able to bind holoTf. Nevertheless, their exact function has not been reported. It was further shown that to internalize iron, a direct contact with holoTf is not necessary, despite the fact that its affinity at the cellular surface is strong. This effect is similar to the iron acquisition mediated by siderophores or other iron-chelating molecules [32].

Recently, the use of holoTf as an iron source was reported in ***Campylobacter jejuni***, one of the most common causes of human diarrhoea. In this bacterium, the iron acquisition process depends on the proximity of a specific receptor named **CtuA** [33], which suggests the presence of **another Fe acquisition system.**

2.2. Gram-positive Bacteria

The cell wall of Gram-positive bacteria is composed of murein, polysaccharides, teicoic acids and proteins that separate the bacterial cytoplasm from the environment. Iron acquisition involves **a binding protein anchored to the membrane (**which is similar to the periplasmic binding proteins: **PBP),** and an ABC transporter **(Figure 2)**.

There is only little information about iron transport in these organisms. Attributable to the collection of the genomic sequences, the genes that encode for Fe transporters were identified. Interestingly, it was found that they are related with those found in Gram-negative bacteria [3].

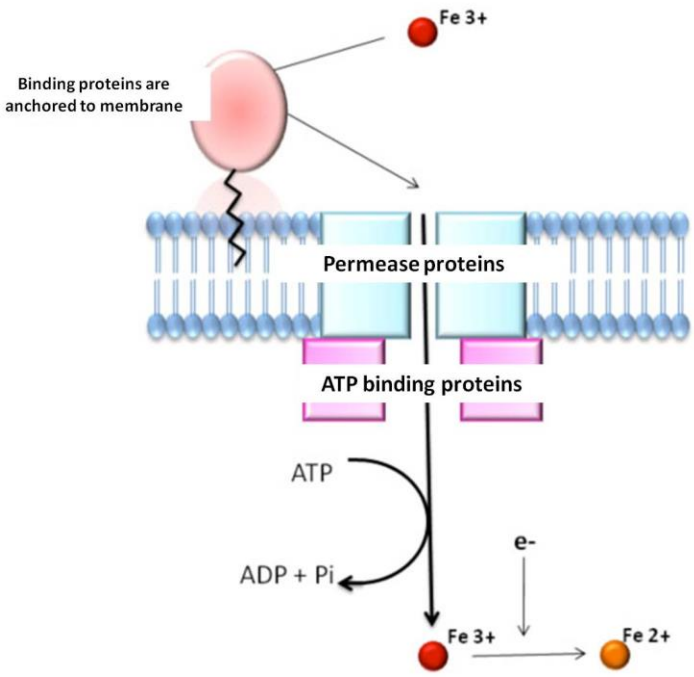

Figure 2. SCHEMATIC REPRESENTATION OF IRON ACQUISITION IN GRAM-POSITIVE BACTERIA.

Acquisition of iron by Transferrin involves a membrane protein and an ABC transporter.

An iron acquisition system has been reported in ***Staphylococcus aureus,*** bacterium that some strains can cause a lethal disease called endocarditis. ***S. aureus*** **requires iron to colonize its host, and in response to the available iron, it regulates genes that ensure the acquisition and metabolism of this**

element. In iron-limiting environments, **Fur, the repressor responsible for iron regulation, coordinates the central metabolic routes, causing the production of a large amount of acid. Consequently, local pH diminishes, resulting in the release of Fe from holoTf.** This is considered a novel mechanism to acquire limited nutrients in the host hostile environment. **In pathogenesis, iron acquisition is extremely important, and accordingly, the systems involved to obtain this vital metal could become most adequate therapeutic targets [**34**].**

A cell-wall based iron acquisition and import pathway was described in *S. aureus***.** The iron-acquisition system involves the **staphylococcal holoTf binding protein StbA,** which is localized in the cell wall and is regulated by exogenous iron levels via the Fur protein. Along with *stbA*, other iron-regulated genes were found. The iron-responsive surface determinant locus or ***isd*** consists of **a membrane transporter, the cell wall anchored Heme-binding proteins, Heme/Hp receptors, two Heme oxygenases, and sortase B,** a transpeptidase that anchors substrate proteins to the cell wall and passes Heme-iron to the cytoplasm. Identification of the *isd* pathway provides information about the roles of the cell wall in bacterial pathogenesis and provides new avenues for therapeutics to combat the rise of antimicrobial resistance in *S. aureus* [22, 35, 36].

A different mechanism was observed in ***Streptococous pyogenes*****, a** coccal β-hemolytic bacterium that produces pharyngo-tonsillitis. **This bacterium obtains iron from holoTf by using the Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in cytoplasmic membrane.** Interestingly, this enzyme is also involved in several cellular events such as the binding to **extracellular matrix proteins** [37, 38].

S. pyogenes possesses a typical ABC transporter, named **MtsABC,** which contains a lipoprotein (**MtsA**). This protein functions as the primary site to bind ions for further transport. **Bicarbonate is required as a synergic anion which promotes the stable union of the Fe^{3+}, as an analogy to human Tf and FbpA in Gram-negative bacteria.** MtsA contains both an ATP binding protein that provides the energy needed for iron transport and an integral membrane protein that permits the release and entrance of Fe, an essential step for bacteria *in vivo* [38].

2.3 Intracellular Bacteria

Intracellular pathogens force the immune system cells to internalize them in vacuoles, and thus they avoid being detected and destroyed. The microorganisms become dangerous when these vacuoles break down and they are in contact with the cytoplasm. Here, they can freely multiply and are able to spread the infection.

Molecules secreted by pathogens as *Listeria monocytogenes* mutants that induce an enhanced or diminished host innate immune response were described, which assess the threat. In this sense, the expression of the **Multi-Drug Resistance (MDR) transporting system controlling the cytosolic surveillance pathway, leads to the production of several cytokines,** including the type I-IFN. **Bacterial MDRs had**

previously been associated with resistance to antibiotics and other toxic compounds. When the expression of MDR increases, the stimulation of the immune system is increased too [39].

Similar results were obtained in an infection with ***Salmonella enterica*** **serovar Typhimurium,** the bacillus responsible for the typhoid fever in mice and for human salmonellosis. In this bacterium, it was observed not only **the relationship with the immunological pathways, but also between the interferon gamma (IFN-γ) activity and iron homeostasis in macrophage, which is what decides the course of the disease.**

IFN-γ is able to reduce Fe availability by a significant reduction of iron uptake via the Transferrin receptor 1 (TfR1), producing the Fe output from Ferritin by expression of the Lipocalin 2 receptor (Lcn2R) and the mobilization of Heme-iron by the Heme Oxygenase 1 (Hmox1), which promotes the siderophore inhibition and catalytic degradation of Heme, respectively. Therefore, bacteria encounter in the host cells an extremely iron-limiting environment, which, as a result, causes a decrease in the infection. The regulation of the intracellular concentration of this element helps to the antimicrobial effects of INF-γ [40].

The importance of obtaining Fe by virulent parasites suggests a potential therapeutic use of compounds that prevent this process. One example is **chloroquine**, a drug that, **by raising the pH, prevents the release of Fe from holoTf**. In addition, the interference with the normal iron metabolism produces a reduction in the number of parasites. **This effect is reversed when FeNTA,** an iron compound that is soluble at neutral to alkaline pH, was added. This highlights the importance of obtaining Fe in these organisms [41].

According to these observations, restricting available iron against invasive pathogens can be an effective strategy to combat infections and enhance host defense mechanisms.

3. Eukaryotes

3.1 Fungi

Fungi effectively regulate the process of iron intake and homeostasis to counteract the restricted iron concentration in the mammalian host, and they also modulate the expression of virulence factors. Most studies have been made in the baker´s yeast ***Saccharomyces cerevisiae,*** which serves as a reference for pathogenic fungi. The investigations are focused on the presence or absence of genes involved in the mechanism that regulate fungal iron intake [42].

Saccharomyces cerevisiae, Candida albicans [43], which produces oral and genital candidiasis, ***Aspergillus fumigatus*** [44], which causes pulmonary aspergillosis and ***Cryptococcus neoformans*** [45], opportunistic pathogen of immunosuppressed patients, **are able to acquire Fe from holoTf.**

C. albicans possesses the ***Ca*FTR2, a high affinity iron permease** which is **homologous to FTR1 of *S. cerevisiae*** to acquire iron in an iron-deficient environment**.** The *C. albicans*, *S. cerevisiae* and *C. neoformans* systems for iron acquisition require the specific holoTf binding to their cell surface, where **a surface reductase** facilitates iron reduction and release. Then a high-affinity transporter-multicopper oxidase (Fet3) transports the ferrous iron into the cell [43, 44, 45]. ***A. fumigatus*** has the possibility of **using siderophores to remove iron from holoTf** in human serum [44]. Both ways of iron acquisition are genetically separated [42].

In fungi, mitochondria are particularly important in the metabolism and homeostasis of Fe. These organelles use large quantities of iron, since **they contain the machinery for both the biosynthesis of Fe-S proteins and Heme,** and for the transport of iron [46, 47].

C. neoformans invades the Central Nervous System (**CNS**), utilizing **a master regulator of iron acquisition from holoTf, which is the most important source of Fe in the CNS. In fact, holoTf is the only iron-carrier protein able to cross the blood-brain barrier** [48]. Cir1 coordinates the Fe acquisition with the expression of fungal virulence factors, such as **melanin and the synthesis of capsule,** just as with growth and **signalling pathways** [49]. *C. neoformans* is able to produce the enzymatic reduction of Fe(III) by secreting reducers, such as the **3-hydroxyanthranilic acid (3AA). This microorganism** can employ melanin to reduce ferric iron and then to transport it. 3AA is essential **for the high affinity iron-acquisition receptor, which is copper-dependent** [46] **(Figure 3).**

When *C. neoformans* was grown under a low iron concentration, 69 genes were overexpressed. On the other hand, 212 genes were highly transcribed under a high iron concentration [50]. The genes that assess the Fe concentration are the **Fe transporter *SIT1*, siderophore transporters *CFT1* (*Cryptococcus* Fe transporter), *CFO1* (*Cryptococcus* Fe Oxidase, an orthologous of *FTR1* from *S. cerevisiae)*, and *CIG1*, that encodes for a glycoprotein inductor of cytokines** [47, 49].

In mouse models of cryptococcosis, Cft1 permease is essential for both to obtain reduced Fe from holoTf and express virulence. A separation of the internalization ways for Fe was demonstrated when mutants in ***CFT1*** gene grew normally in the presence of various Fe sources. On the other hand, **the mutant lacking *CFT1* showed diminished the Fe concentration in the infected mice brain. This result is related with a defect in the utilization of holoTf.**

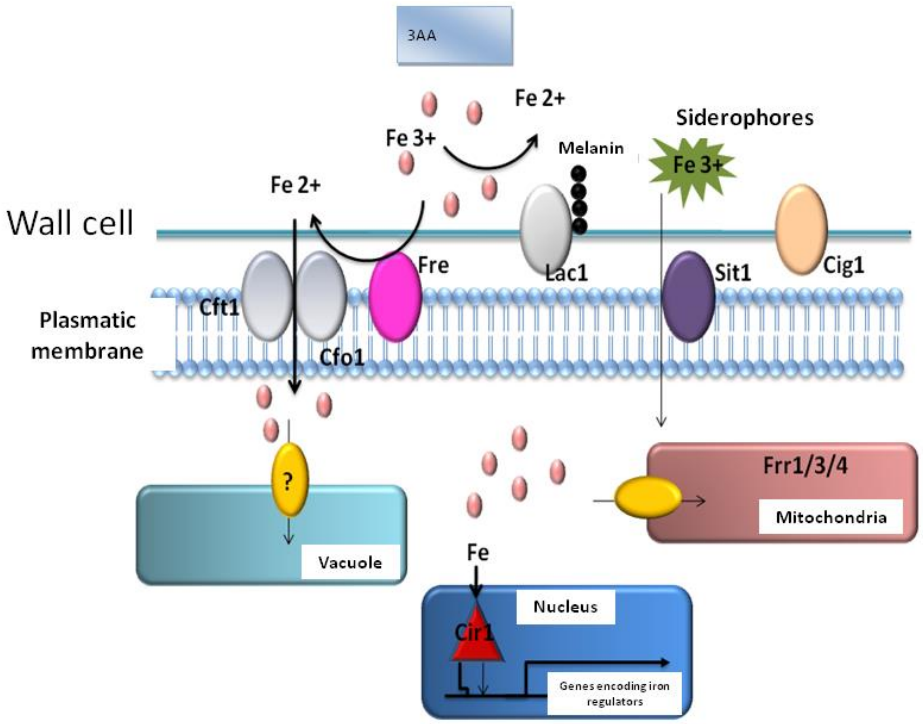

Figure 3. IRON ACQUISITION AND HOMEOSTASIS IN *C. neoformans*

Reductases in the cellular surface (Fre) reduce the ferric into ferrous iron. This is transported to the interior by the permease (Cft1) and ferroxidase (Cfo1) in the plasmatic membrane (PM). The extracellular reducer, 3-hydroxyantranilic acid (3AA), and melanin are involved in the ferric iron reduction and the siderophore transport Sit1. Cig1 from the cell wall (CW) and the mitochondrial proteins Fr1, 3, and 4 participate in iron acquisition and homeostasis. The master iron regulator, Cir1, controls genes transcription and exerts its influence on gene expression.

When the iron bioavailability is low, **some species of fungi synthesize and secrete siderophores,** which are specific chelants of ferric Fe. Despite the fact that ***S. cerevisiae, C. albicans* and *C. neoformans* do not synthesize siderophores, they can sequester Fe bound to xenosiderophores, i.e., those secreted by other organisms** [51]. ***S. cerevisiae*** scavenges Fe bound to exogenous siderophores by the Fe reducer system, or by membrane transporters that function in response to diverse substrates [51].

The mentioned iron-acquisition mechanism is relevant in the case of **desferrioxiamine, which is prescribed to control the infection of pathogenic fungi, while, in reality, the infection is exacerbated.** This phenomenon was observed in an experimental model of mucormycosis [52]. In ***C. neoformans* and *S. cerevisiae*,** the transporter for desferrioxiamine B siderophore is encoded by the *SIT1* gene [53], whose product does not seem to play a significant role in host tissues.

Other fungi, such as ***A. fumigatus,*** synthesize siderophores to take up iron from holoTf by using **transporters in the cellular surface** [49, 53, 54, 55, 56].

More research is needed to understand **which iron source is preferred by pathogenic organisms and which mechanisms they employ to acquire iron during pathogenesis.** In the case of the transcription factor Cir1, the most challenging task remains in to fully understand the Fe regulation mechanism, its metabolic functions, and the synthesis of the virulence factors. This information, in turn, may shed light on the pathogen's nutritional adaptation to the host environment during the colonization of tissues.

3.2 Protozoa

In this chapter, we will now to consider the studies that describe the reception and endocytosis of holoTf in various protozoa, except ***Entamoeba histolytica, Leishmania spp., Trichomonas vaginalis, Tritrichomonas foetus*** and ***Toxoplasma gondii.*** These parasites will be discussed in detail in future chapters. A recent review is found in: [57].

Trypanosoma brucei is a parasitic protist that causes the sleeping sickness. It requires an insect vector (tse tse fly) for its transmission to human beings and some species of animals. During its blood stage, **holoTf is the only Fe source for *T. brucei* in the mammalian host.**

The receptor that internalizes holoTf in *T. brucei* has been identified. This receptor is present in the flagellar pocket [58, 59], and **consists of heterodimers of the associated expression sites encoded by the genes *ESAG6* and *ESAG7*.** The *ESAG* genes are associated with a site of variable expression surface **in a subtelomeric region,** a genome region with great plasticity, which presents the accumulation of species-specific sequences [60]. The protein anchored by GPI, ESAG-6 [61], is of great importance in the parasite´s adaptability to its host [62].

Although t**he *ESAG6* and *ESAG7* genes** differ in expression sites, the sequences between species resemble each other. **Small changes in the sequence allow *Trypanosoma brucei* to adapt to different mammalian hosts by modifying its host receptor specificity** [63]. Consequently, this endows the parasite with the ability to infect a wide variety of mammals. **These receptors present both low and high affinity simultaneously** [62, 64]. Protein ESAG6 exhibits a glycosylphosphatidylinositol (GPI) anchor site at the C-terminus, linking the heterodimer to the membrane in the flagellar pocket. Interestingly, its structure showed that GPI lipids are relevant to their location in the flagellar pocket [58].

When the regions of both intra- and inter-species of *Trypanosoma* concerning to the genetic variability of the holoTf receptor were analyzed, it was observed that **the holoTf receptors of *T. evansi* are diverse genetically and correlate with many hosts. *T. evansi* and *T. brucei* have some unique variations in the *ESAG6* genes and in the binding sites to Tf.** This could account for the difference in the pathogenesis

between these two species in distinct hosts. The hypervariable region of the receptor is not involved in the holoTf binding. However, this region has a role in the antigenic variation of the receptor's surface region, evading the immune reactions of the host [65, 66].

Trypanosoma brucei* and *T. congolense (causal agents of severe anemia in cattle), scavenge **Fe from the host holoTf by receptor-mediated endocytosis.** This process is conducted in the specialized nutrient-uptake region of these parasites, commonly named **flagellar pocket.** The endocytosis process may be **dominated by clathrin vesicles** [67]. It was suggested that both the holoTf receptor in *T. brucei* and the GPI anchorage proteins are endocytosed by a clathrin-mediated mechanism, **based on ultrastructural and biochemical studies** [59, 67, 68, 69, 70, 71]. This endocytic process involves the formation of a **heterodimeric complex formed by the soluble protein encoded by the gene *ESAG-7* and small coated vesicles of 100-150 nm diameter.**

Endocytosis in *T. brucei* is regulated during the parasite´s development; it is more active during the infective stage in the mammalian host, where apparently this process is crucial **for avoiding drugs, evading the immune system and scavenging nutrients.** The low-specificity receptor for mammalian Tf provides the parasite the opportunity to increase its number of hosts to other vertebrates, such as reptiles [64, 72].

The concentration of anti-Tf receptor (anti-*Tb*TfR) antibodies present during the chronic infection with *T. brucei* is very low to avoid the parasite can obtain Fe from its host; therefore, the response to the infection is rather mild [73].

***Tb*RAB5 and *Tb*RAB11 GTPases are highly expressed in *T. brucei*.** Rab proteins are involved in the recycling of the GPI anchorage proteins, the VSG protein and TfR and also in the trafficking of internalized anti-VSG antibodies and Tf [67, 74]. During the cellular development of other cells, the protein **Rab11 exhibits various specialized functions.** In mammals, for example, it regulates the calcium concentration associated with exocytosis in neuronal cells [74].

***Tb*RAB5A and *Tb*RAB5B (Rab5 isoforms)** have several functions at the stage of the mammalian host bloodstream, when clathrin-mediated endocytosis is involved. At the procyclic stage, the two isoforms occupy the same compartment and have similar effects in the endocytosis by fluid phase. **The differences in the endocytosis regulation between the two stages of the parasite's life cycle reflect the necessities to survive in two different hosts: insects and mammals** [74].

Recently, **in *T. cruzi,*** the protozoan causal agent of the South American tripanosomiasis or **Chagas disease, investigations revealed** the **presence of clathrin** [75], in a **vestigial endocytic process** characteristic of the flagellar pocket. **This organism later evolved a more efficient endocytic pathway, which is clathrin-independent, in the cytostome/cytopharynx organelle.** This organelle is a deep invagination in the cytoplasm from the plasmatic membrane to the nucleus [75, 76].

This new endocytic process, which is cholesterol-dependent, is more efficient for the Tf internalization, transport and storage in **reservosomes.** These organelles are pre-lysosomes where apparently the vesicles without cover do not bind. **Reservosomes represent the late endosomes** [75, 77].

Adaptation of *T. cruzi* to the host-parasite relationship plays a role in **the selective process that leads to the polarization of the holoTf internalization toward the cytostome.** Therefore, this adaptation renders the transport of molecules to cytoplasmic targets more efficient [76].

After endocytosis, the Tf-TfR complex is processed in lysosomes. Tf is consequently degraded to provide Fe to the parasite. The receptor is recycled again to the flagellar pocket in approximately 11 min [78], **by the action of the cysteine-protease rhodesain (a cathepsin L-like) and the *Tb*CAT-B (cathepsin B-like enzyme).** This was concluded in a study where the proteins were inhibited. As a result, the parasites showed a defective degradation of the host proteins, including Tf [78, 79].

Iron acquisition is an essential process for pathogens. This process has evolved and has become specialized for the distinct hosts through evolution, regarding to the infection target and type of molecules it faces. It is necessary to perform more studies to better understand the different mechanisms of iron acquisition in parasitic organisms. This knowledge will help to vaccines production and to find therapeutic targets to combat the infection.

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Chapter 14

The two faces of Lactoferrin in its interaction with pathogenic microorganisms

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Abstract

In contrast to the large variety of microorganisms that use iron (Fe) from Transferrin (Tf) and Hemoglobin (Hb) for their growth, only virulent pathogenic microorganisms that exclusively reside in host mucosae or infected sites use Fe from Lactoferrin (Lf).

Once again, we direct attention to the Lf protein. In the preceding chapters, the Lf protein is described as multifunctional; it can exert adverse effects on microorganisms, and depending on the environmental and external factors, Lf can behave differently. By the end of this chapter, the concept of a "moonlighting protein" should be clear. This concept is used for proteins, such as Lf, that exhibit two contradictory functions in microorganisms; *e.g.*, holoLf (with iron) is a source of Fe that can sustain the growth of pathogens, but apoLf (without iron) can inhibit their growth.

In the interaction of Lf with pathogens, this protein shows three different activities: growth stimulation when is iron-charged, and microbiostatic or microbicidal when is iron-lacked. The first antimicrobial property discovered for Lf was its ability to sequester the Fe necessary for bacterial pathogens, thus inhibiting bacterial replication, which was recovered when Fe was added. Subsequently, the ability of microorganisms to develop a system that can effectively use host Lf as Fe source for their growth was described. Later, the direct interaction of Lf with the bacterial surface and the damage of vital structures were described as direct microbicidal effects.

Research in the Lf field has demonstrated that Lf also have an indirect microbicidal effect by stimulating the immune response and increasing the effect of proteins with microbicidal activity. ApoLf acts synergistically with antibiotics against drug-resistant microorganisms, making it an attractive molecule for clinical use. For this reason, Lf represents an expanding field of research with very interesting potential applications with regard to new technologies that can be applied to emergent disease treatments.

Keywords: bacterial growth, iron, microbicidal, microbiostatic, lactoferrin, pathogens, synergy, innate immunity.

1. Introduction

The majority of iron (Fe) found in the human body is bound to proteins, such as Transferrin (Tf) in the serum and lymph, Hemoglobin (Hb) and Ferritin (Ft) inside cells, and Lactoferrin (Lf) in the mucosae and extracellular fluids. In this chapter, we describe the interaction between Lf and pathogens.

In chapter seven, we mentioned that Lf is found in two forms: the iron-bound (holoLf) and iron-free (apoLf) states. Therefore, interactions between pathogens and both forms of Lf can occur. In most cases, holoLf is used as an iron source by pathogens to maintain and ensure the successful infection of the host. In contrast, apoLf acts as a microbiostatic or microbicide.

Due to Fe is a cofactor for several enzymes and catalyzes vital reactions in energetic metabolism, and DNA synthesis in all cells [1, 2], any microorganism that enters a host cell needs to obtain Fe from the available sources to adhere, multiply, establish, colonize, invade, and cause damage to the host. Then, if a microorganism possesses diverse systems to obtain Fe, it can multiply in different niches [3]. These Fe acquisition mechanisms are crucial for microbial establishment and disease progression in the host [4].

The different mechanisms used by microorganisms to acquire Fe are classified in the following manner:

a. Fe acquisition from organic material

b. Production, excretion and/or siderophore uptake

c. Protease production

d. Fe-reductase expression

e. Phagocytosis of bacterial, fungal, and host cells

f. Expression of chelants

Since **Lf is a mammalian iron-containing protein that interacts directly with the membranes of certain pathogenic microorganisms,** this interaction is likely to have utility for both, host and pathogen. Therefore, although the microbicidal effect of Lf and its peptides has been carefully demonstrateded, **pathogens have developed various strategies that protect themselves against the Lf´s microbicidal action.** On the other hand, since Lf can sequester Fe (holoLf) to deprive pathogens of this vital element, **some microbes have developed mechanisms to use the Fe contained in Lf to maintain their growth.**

Although certain researchers may not think it possible, opposing effects of Lf do occur. It is very important to maintain Fe homeostasis in the organism, and when discussing the role of Lf in the cell, its microbicidal capacity is the commonly included. However, highly virulent microorganisms have developed various mechanisms to steal the Fe from holoLf. As previously described, in mammals, pathogens that use Fe

from Lf are exclusive to their hosts [5]**.** The study of both effects of Lf on pathogens is a challenge in biological research.

2. Bacteria use holoLactoferrin iron for their growth

It is known that the **Fe contained in holoLf is used by bacteria for their growth** [6], and **one of the mechanisms utilized is through siderophores (their own or those of other microorganisms), which remove the Fe from holoLf).** Siderophores are low-molecular-weight compounds (less than 1 kDa) with high affinity for Fe that can effectively compete with host proteins for Fe; thus, a siderophore wins to Lf in the competition for Fe**. Other mechanisms are the use of holoLf receptors, and the secretion of proteases that cleave holoLf** [5, 7]. Certain pathogens use a combination of several of these mechanisms to acquire Fe; these microorganisms include bacteria, fungi and certain protozoa.

The use of Fe from holoLf by bacteria has been well studied in the ***Neisseria*** genus. ***N. gonorrhoeae*** is the causal agent of **gonorrhea,** which is the most important sexually transmitted disease worldwide. The disease appears as acute urethritis in male and cervicitis in female. Approximately 5% of infected males and 50% of infected females are asymptomatic. In the male, this disease can cause epididymitis and prostatitis, but in females it causes pelvic inflammatory disease with infertility and ectopic pregnancy [8]. **This bacterium lives in the genital mucosa, and holoLf provides the Fe for its growth.**

To use holoLf, *Neisseria* utilizes membrane proteins that are capable of binding to the charged Fe-Lf complex. **The holoLf receptor (LfR) or holoLf binding protein (Lbp) is also found in *Neisseria meningitidis***. This bacterium causes **septicemia and meningitis,** is highly virulent and is an important cause of child mortality [9]. **Lbp was also found in the commensal species of the genus** [10], indicating that both pathogenic and non-pathogenic bacteria require Fe for their establishment. [11]. The first protein found to be involved in Lf recognition was a 105-kDa protein from *Neisseria* known as **LbpA**, **which is homologous to the TbpA,** protein used by *Neisseria* to bind holoTransferrin (holoTf). Another known protein utilized for Fe acquisition from holoLf is **LbpB** [12, 13].

Moraxella* (*Branhamella*) *catarrhalis, a pathogen that causes **otitis media** in infants and lower respiratory-tract infections in all ages, utilizes a similar mechanism and proteins to *Neisseria* for Fe acquisition from holoLf. In addition, **in Fe stress conditions, *Moraxella* induces the expression of a receptor of 100 kDa in size that is useful for obtaining Fe** [14].

Helicobacter pylori, a bacterium that causes **gastritis and ulcers and has been linked to gastric cancer** [15], **can survive with the Fe that host holoLf supplies in the gastric mucosa.** The bacterium uses a membrane receptor to bind the charged protein and obtains Fe in this manner. ***Campylobacter jejuni***, which is related to **diarrhea in inhabitants of developed countries,** can survive on the Fe provided by holoLf and

other transferrins [16, 17]. **Enteropathogenic *Escherichia coli*, the dental pathogens *Prevotella* spp.** and ***Actinobacillus actinomycetemcomitans*, the nosocomial pathogen *Klebsiella* spp., and *Vibrio cholerae*, the causal agent for pandemic cholera,** use the Fe supported by ferric proteins in the human body, including **holoLf** [18, 19, 20].

The Gram-negative bacteria mentioned previously can steal the Fe from holoLf for use in their growth. To accomplish this task, these bacteria use proteins localized at their external membranes that have a high affinity for holoLf. **Initially, holoLf is bound by the union protein LbpB on the surface of the external membrane, which is an irreversible interaction.**

It has been documented that LbpB facilitates the binding of holoLf to LbpA, an integral membrane protein with high affinity for Lf that undergoes an irreversible reaction with the carboxyl lobule of Lf, inducing a conformational change and the subsequent release of Fe^{+3}, which is transported to the periplasm and provided to the periplasmic binding proteins that go to the external region of the internal membrane.

It has been postulated that **Fe crosses the periplasm of Gram-negative bacteria by diffusion,** using a site generated by permeases localized at the inner membrane. **This transport requires a proton motive force and an integral inner membrane complex containing TonB, ExbB and ExbD.**

Fe transport across the inner membrane is catalyzed by an ABC (ATP-binding cassette) transporter; these transporters are frequently used for nutrient acquisition in both Gram-negative and Gram-positive bacteria [5, 13]. **The Fe is incorporated into bacterial synthesis or stored in a Ferritin-like complex.** Figure 1 outlines the proposed model of Fe acquisition from holoLf by Gram-negative bacteria.

In the case of Gram-positive bacteria, which only contain the permeable cytoplasmic membrane, holoLf has directly access to this membrane. HoloLf is recognized by **LbpA or a similar receptor** on the surface, which allows **only Fe^{+3} to pass and then transports it to the cytosol** using a similar mechanism to that observed in Gram-negative bacteria. Finally, **Fe^{+3} is reduced to Fe^{+2} and is used in various cell processes,** including oxidation-reduction, as a component of structural proteins, as cofactor for different metabolic reactions, as genic regulator, and in DNA biosynthesis.

It has been shown that ***Corynebacterium diphtheriae***, the causal agent of diphtheria in children, grows in the presence of holoLf as its only Fe source. ***Gardnerella vaginalis***, which causes bacterial vaginitis in women of reproductive age, also uses Fe from holoLf. To accomplish this feat, **these bacteria have a 120 kDa protein that can assess whether the bacterial cell is experiencing environmental Fe stress** [21].

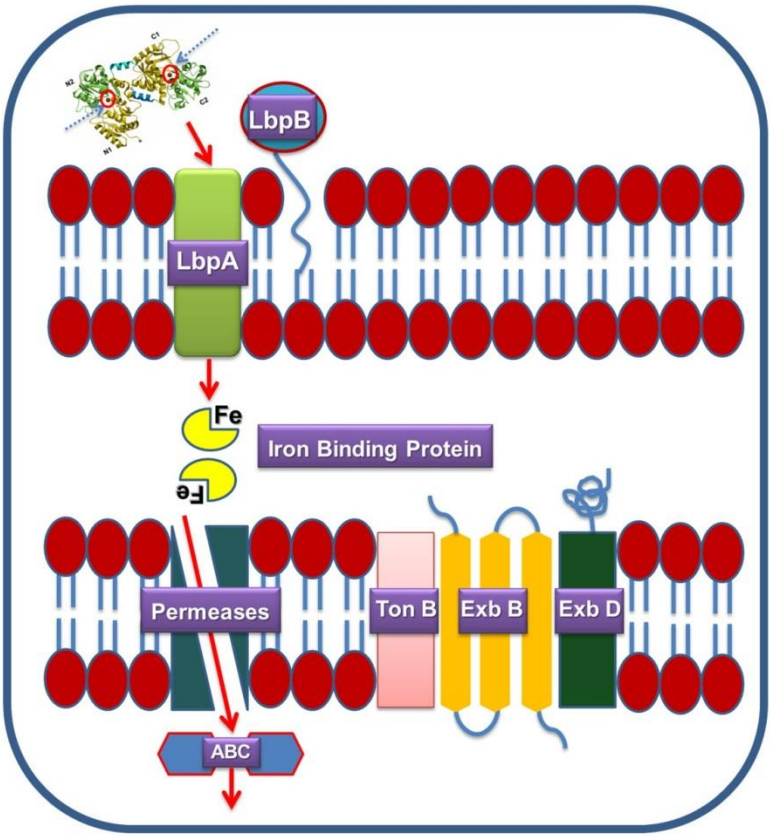

Figure 1. A PROPOSED MODEL FOR THE IRON ACQUISITION SYSTEM USED BY GRAM-NEGATIVE BACTERIA TO OBTAIN IRON FROM HOLOLF

Certain beneficial bacteria that maintain the intestine, such as ***Bifidobacterium*** and ***Lactobacillus***, can also use the Fe from holoLf. Moreover, it has been proposed that **prophylactic therapy with apoLf can inhibit the growth of pathogens that cause diarrhea** and, on the other hand, that **holoLf can support the growth of commensal bacterial and maintain the flora balance** [22]. Figure 2 shows the proposed mechanism for Fe acquisition from holoLf used by Gram-positive bacteria.

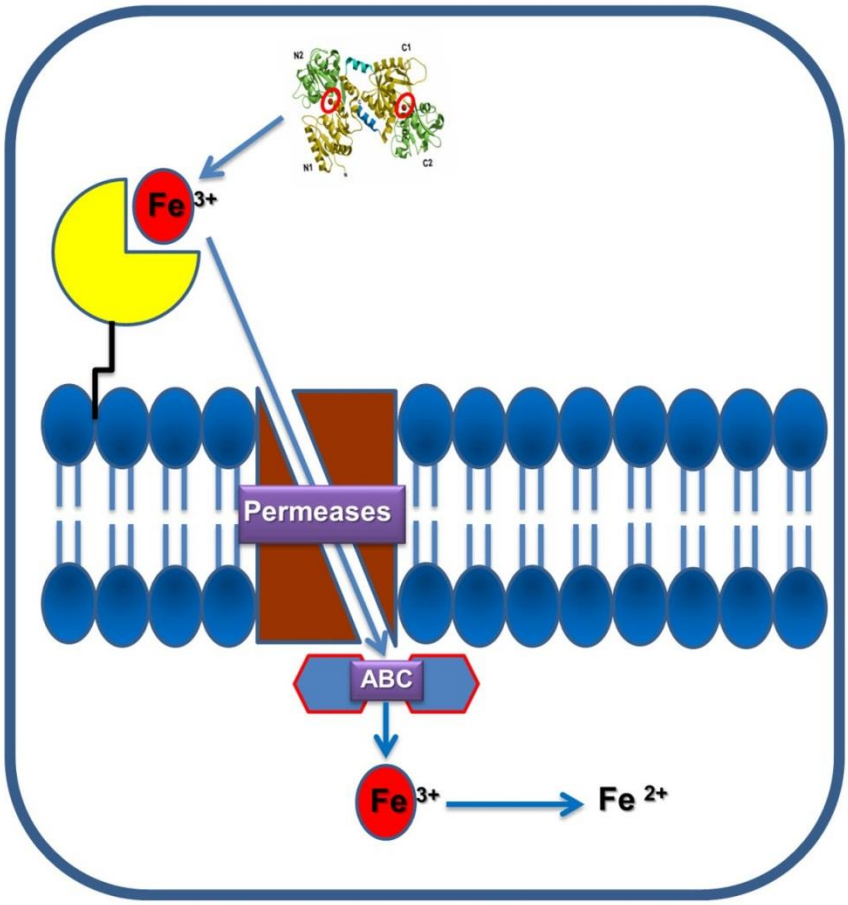

Figure 2. A PROPOSED MECHANISM FOR IRON ACQUISITION FROM HoloLf BY GRAM-POSITIVE BACTERIA.

3. HoloLf as an iron source for fungi and protozoa

Recently, it has been demonstrated that **holoLf promotes the growth of *Candida* spp., whereas apoLf has a fungicidal activity if the culture medium does not contain Fe** [23]. Many different mechanisms for Fe acquisition in fungi have been described; however, Fe acquisition from holoTf and holoLf has not been studied in pathogenic fungi, with the exception of the work described in ***Candida* spp.** [23, 24].

The use of Fe from holoLf by bacteria has been described in many genera; however, in fungi or protozoa the reports are scarce [3, 7]. A recent review about the utilization of host holoLf as an iron source by parasites is found in [25]. ***Trichomonas vaginalis*** is**,** a parasite that infects genital mucosae and can cause human trichomoniasis, the most important non-viral and curable sexually transmitted disease in sexually active young women. ***T. vaginalis* infection is associated with perinatal complications and susceptibility to HIV infection if individuals are exposed to both HIV and pelvic inflammatory disease** [26]. It has been reported that **receptors in the membrane that have an affinity for holoLf exist in this parasite,** but

the mechanism by which it endocytoses this ferric protein remains unknown [27]**.** In the related parasite, ***Tritrichomonas fetus***, a **bovine genital pathogen, the proposed mechanism involves the use of membrane receptors, endocytosis mediated by a receptor, and a protease that can degrade holoLf** [28, 29].

Parasites that cause leishmaniasis, which is a zoonotic disease caused by different **species of the *Leishmania* genus, can use Fe from holoLf for their growth.** The clinical features of this disease range from **skin sores (ulcers),** which erupt weeks to months after the person affected is bitten by sand flies, **to fever and damage to the spleen and liver;** therefore, **these protozoa could acquire Fe from proteins either from human or insect** [30].

Interestingly, the binding proteins that recognize holoLf **(Lbps) are versatile, in that they recognize not only holoLf, but also Transferrin (holoTf) and serum albumin** [31, 32]. A work published in 2002 demonstrated a mechanism used by ***L. chagasi*** to obtain Fe from holoLf and holoTf [32]. **Apparently, this protozoan has a membrane protein complex that reduces the Fe^{3+} and a second protein that transports the Fe^{2+} into the cell** [32].

As the iron acquisition systems developed by pathogens are very important to elucidate the initial process of infection, our research group studies the ability of the parasite ***Entamoeba histolytica*** to use several human proteins that contain Fe, such as Lf, Hb, Tf, and Ft. This protozoan is the causal agent of amebiasis, and it requires a high concentration of Fe for growth (>60 μM), a characteristic that is similar to other primitive eukaryotes. In chapter 17, we detail how holoLf supports the *in vitro* growth of *E. histolytica* and the endocytic mechanism that the amoeba utilizes to internalize this ferric protein.

4. The other side of Lactoferrin: microbicidal action

We described above how bacteria, fungi and protozoa extract Fe from holoLf to support their growth. In this section we will discuss the other action of Lf on microorganisms, *e.g.* apoLf microbiostatic and microbicidal effects, and the mechanisms used by apoLf to kill pathogens. The microbiostatic effect was the first biological property attributed to Lf; this property is due to the iron chelating activity of Lf. However, we now know that Lf has other functions involved in the host's defense.

There exist five basic mechanisms for Lf involvement in the host defense against a large variety of Gram-positive and Gram-negative bacteria, and they can be summarized as follows:

4.1. Microbicidal mechanisms of Lactoferrin

A. *Iron binding ability*. Via its high affinity for Fe, it has been postulated that apoLf sequesters Fe, making it unavailable to pathogens and killing them by **nutrient deficit** [25].

B. ***Interaction between apoLf and structural molecules in the bacterial membrane and subsequently bacterial lysis.*** ApoLf has a high isoelectric point, and the metal-free **apoLf form exposes positively charged amino acids,** which allow it to interact with negatively charged components of microorganisms. After such interactions, there is a remotion of bacterial structural components and a loss of the selective permeability of the bacterial cell [33].

C. ***Inhibition of pathogen adhesion to the host cell.*** ApoLf blocks the sites where the pathogen will adhere. This blockage prevents the pathogen from initiating infection [34].

D. Inhibition of bacterial colonization factors caused by the proteolytic activity and hemolysin inhibitor effect of apoLf. **ApoLf degrades bacterial proteases,** preventing their effect on the host. Additionally, it inhibits the action of bacterial hemolysins [35, 36].

E. Inhibition of bacterial biofilm formation. ApoLf interferes with the signals leading to bacterial movement for biofilm formation. Alternatively, Lf can inhibit bacterium-bacterium adhesion or bacterium-substrate adhesion [37, 38].

As mentioned, the capacity of Lf to inhibit bacterial growth *in vitro* was the first function described. This phenomenon was first explained by the Lf capacity to sequester Fe from bacterial pathogens, thus inhibiting bacterial growth [39]. **The Fe deficit can be reversed by the addition of Fe, and therefore, apoLf is bacteriostatic** [40].

Subsequent research has shown that Lf can be microbicidal, which requires binding to the pathogen surface (bacteria, fungi, virus and protozoa; this effect includes the basic N-terminus of Lf) [41]. Figure 3 depicts the microbicidal action of Lf on **Gram-negative bacteria**, and Figure 4 depicts its action on **Gram-positive bacteria.**

4.2. Bactericidal activity

Lf has been reported to interact directly with microbial membranes, **altering the membrane permeability** through the dispersion of membrane components, such as lipopolysaccharides (**LPS) in Gram-negative bacteria and lipoteichoic acid in Gram-positive bacteria,** and **leading to death of the organism** [42,43,44].

The anti-aggregative effect of Lf is similarly interesting because it influences the inhibition of biofilm formation of ***Pseudomonas aeruginosa*** and ***Shigella flexneri*** [38, 45], which are the most important bacteria in **cystic fibrosis** patients and intensive care units; *S. flexneri* produces **dysentery** in patients.

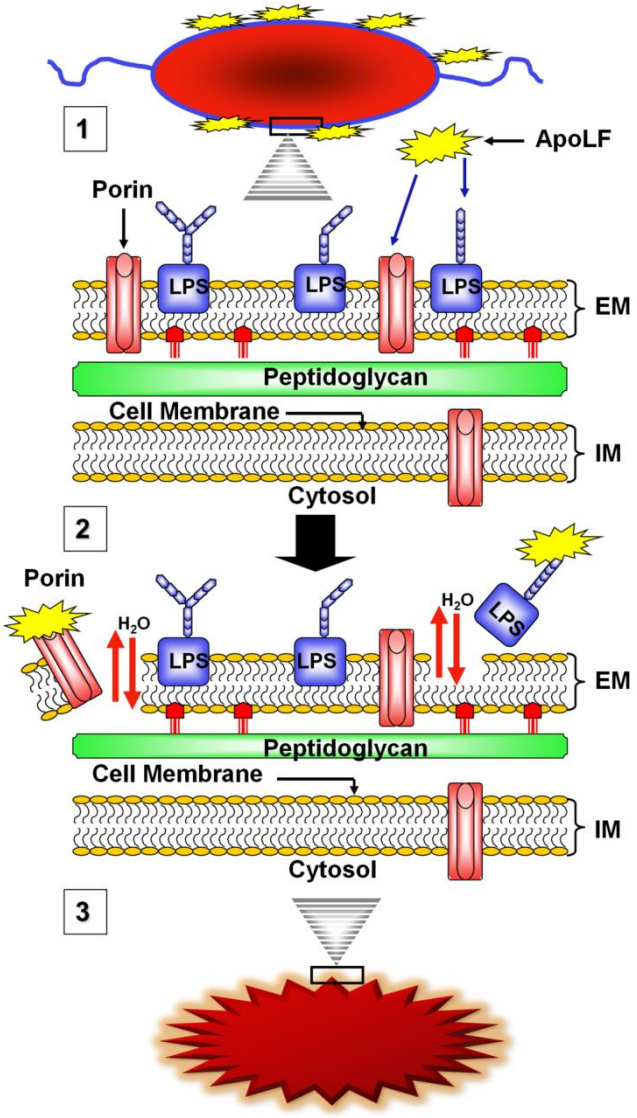

Figure 3. A PROPOSED DIRECT BACTERICIDAL MECHANISM OF ApoLf ON GRAM-NEGATIVE BACTERIA.

Gram-negative bacteria have lipids, such as lipopolysaccharides (LPS), and proteins, such as porins, in their external membrane (EM). 1) The protein apoLf has an affinity for the A lipid from LPS and for porins. Once apoLf binds to, it is capable of 2) separation from the external membrane, causing disorder, destabilization and the generation of holes and 3) loss of selective permeability and cell surface damage. Finally, the bacteria are lysed and die.

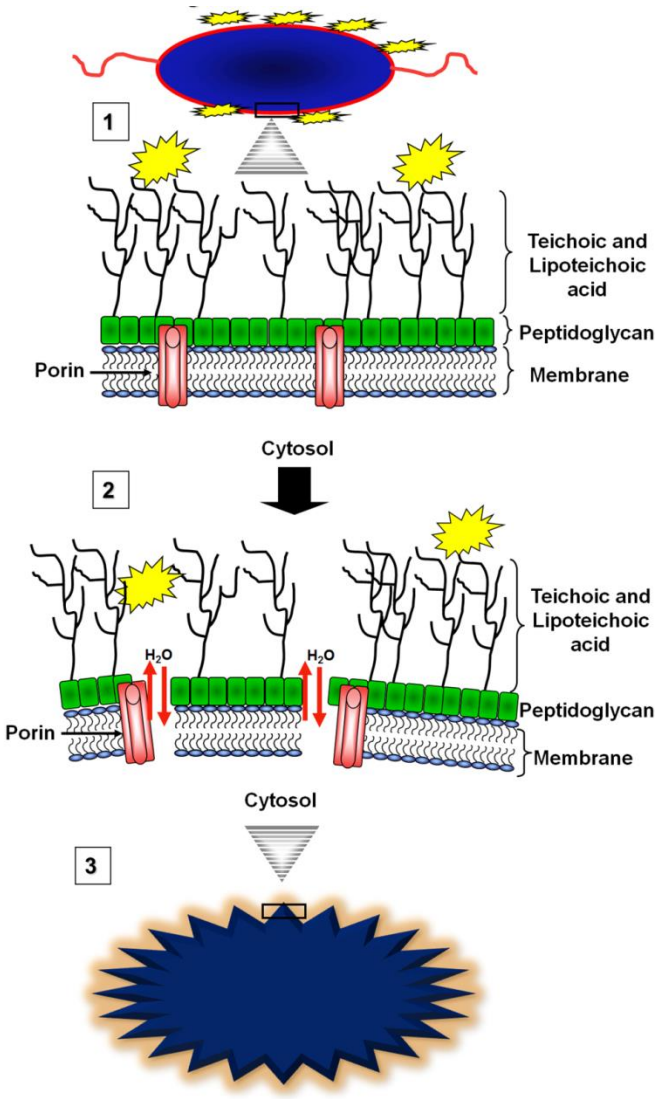

Figure 4. A PROPOSED DIRECT BACTERICIDAL MECHANISM OF ApoLf ON GRAM-POSITIVE BACTERIA.

The Gram-positive bacteria have lipoteichoic acids in their cell wall. 1) ApoLf has an affinity for these compounds and interacts with them. 2) The lipoteichoic acid is separated from the membrane. 3) This separation allows access of apoLf to the phospholipids, for which apoLf has an affinity, causing the lipoteichoic acid and phospholipid to be released from the membrane and the loss of selective permeability. The cell surface is damaged and finally, the bacteria are lysed and die.

Lactoferrin can also amplify apoptotic signals in infected cells, causing the pathogen to be eliminated and allowing for exposure to host defense mechanisms or natural removal.

The bactericidal effect of Lf was originally described in 1977 against ***Vibrio cholerae*** and ***Streptococcus mutans*** [39], the latter bacterium being the causative agent of **dental caries.** The bactericidal mechanism was clarified by studies showing that **Lf can break or permeate the bacterial membrane** [46]. Other studies established that basic peptides localized in the N-terminal portion of Lf, called **Lactoferricins (LFcins),** are more potent than the native protein, but whether the mechanism of damage is similar to the Lf remains unknown [47].

The study of Lf has demonstrated that **the bactericidal effect** of this protein occurs through **an iron-independent mechanism,** and Lf can interact directly with microbial membranes, causing the release of molecules and the pathogen lysis. **In Gram-negative bacteria, such as *Escherichia coli*, Lf binding to lipid A of LPS and to porins has been reported to cause bacterial death** [48, 49]. Recent research has demonstrated that Lf can also have an antibacterial effect via **interaction with the bacterial cell wall,** destabilizing it and causing death [33]. By the bactericidal mechanism, **Lf displays a vital role in the protection of newborns against gastrointestinal infections** [50, 51].

It is also interesting that some of the fragments of Lf produced during the gastric digestion of this protein in the intestines of newborns are capable of fixing Fe. **These fragments are stable at the acidic pH of the stomach, and have been found in the stools of children fed with breast milk.** This observation indicates that biological activity can occur in all gastrointestinal tract [52]. Fortunately, **peptides that exert this natural effect can be synthesized *in vitro* rapidly and at low cost from bovine Lf.** These peptides are harmless, and their microbicidal activity is more effective than those of the native molecule because their net cationic charge is higher than that of Lf.

4.3. Fungicidal activity

Microbicidal activity of Lf is also observed against ***Candida albicans*, an opportunistic fungus that affects immunocompromised people**. As described in bacteria, researchers first thought that the candidacidal activity of Lf was due to sequestration of Fe and deprivation of this element from the fungi. However, **a direct interaction between Lf and the cell wall of *C. albicans*** has been shown [53]. The candidacidal mechanism is related to the alteration of cell membrane permeability, ion discharge and water inlet [54].

Lf-derived peptides alter the membrane permeability of *C. albicans*, affecting hyphal growth in several azole-resistant strains. Additionally, it has been demonstrated that Lf acts against fungi of the ***Trychophytum*** genus, an opportunistic skin pathogen of immunocompromised patients [55, 56].

4.4. Antiviral activity

In viruses that affect the human health, apoLf inhibits viral replication in the early stages of infection as a result of the **binding of Lf to viral particles or to the cell receptor-type glycosaminoglycans that the**

virus uses to enter the host cell [57]. Additionally, antiviral activity of Lf has been described in *in vivo* models, and the effect has been proposed to occur by direct interaction of Lf with the virus, affecting their influence in stimulating the immune system. **Viruses sensitive to the effect of Lf include the causal agents of AIDS, hepatitis C, polio, herpes, and enteroviruses** [58].

In the case of ***Cytomegalovirus***, it has a characteristic ability to remain latent within the body over long periods and can cause pneumonia, hepatitis, gastric ulcers, retinitis, encephalopathies, and endocrine changes in immunocompromised patients. It has been documented that **Lf can inhibit the entry of the virus into macrophages in *in vitro* assays, and prevent viral replication once the cells are infected** [59, 60].

Furthermore, it has been found that **native Lf and its peptides act synergistically with the antiviral agent acyclovir to inhibit viral replication in infected cells** [60]. It has recently been demonstrated that **Lf binds to the E2 glycoprotein of the viral envelope of hepatitis C,** which is the causative agent of chronic hepatitis, hepatic cirrhosis and hepatocarcinoma. These new findings offer the possibility of new research into the prevention of this infection, which afflicts more than 170 million people worldwide [61].

Lf also binds glycosaminoglycans and other host cell molecules, blocking the sites that herpes simplex type I, papilloma, HIV, hepatitis B and C, and adenoviruses use for access to the cell. Through a combination of inhibition of viral replication, receptor blocking of viral endocytosis to the cell and likely other events, Lf protects the host cell from viral infection [62, 63, 64, 65, 66]. Figure 5 depicts Lf action on viruses.

4.5. Parasiticidal activity

In contrast to the large number of studies that describe the bactericidal activity of apoLf, there are few studies in protozoa. A recent review about the parasiticide action of apoLf is found in [67].

Toxoplasma gondii is sensitive to Lf, and it has been suggested that **this protein binds the negative charge of the tachyzoite's membrane.** However, the molecules involved in the parasiticidal effect are unknown [68, 69]. Moreover, **the growth of *T. gondii* in macrophages is inhibited by Lf,** and this effect is dose-dependent, whereas the penetration of *T. gondii* into the host cell is not affected by Lf. In a toxoplasmosis model in cats, it has been found that **orally or intraperitoneally administered Lfcin B induces resistance to sporozoite infection** [70].

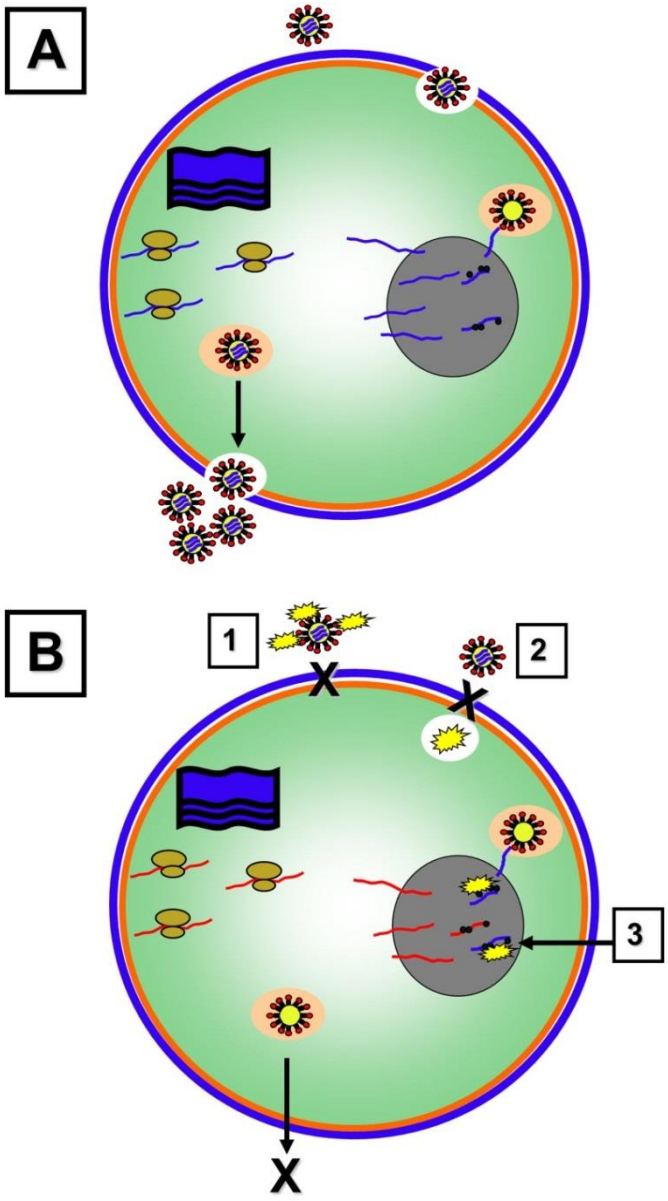

Figure 5. THE VIRUCIDAL MECHANISM OF LACTOFERRIN.

Viruses are particles that do not growth through cell division because they are acellular. Instead, they use the transcription and translation machinery and metabolism of the host cell to produce multiple copies of themselves and infect many adjacent cells (scheme A).

Lf binds proteins on the viral surface, such as polysaccharides (glycosaminoglycans) and cell membrane structures (acting as a signal modulator in the cell communication process). Viruses use proteoglycans, such as heparin sulfate, to bind and be internalized; however, Lf can 1) bind viral particles that interact with proteoglycans, 2) block cellular receptors (binding glycosaminoglycans, such as heparin sulfate) and disrupt virus entry. 3) Alternatively, Lf can inhibit viral replication in the host cell by an unknown mechanism. Both apoLf and holoLf have these antiviral properties.

Plasmodium falciparum, the parasitic protozoan that causes **malaria,** a disease causing acute anemia, is also affected by the action of Lf, and it has been reported that this effect occurs following interaction between Lf and the parasite membrane [71]. In the case of ***Giardia lamblia,*** a parasite of the small intestine that frequently infects children, Lf and Lfcin B have a dose-dependent **giardicidal activity that is inhibited by divalent ions, such as Mg^{2+}, Fe^{2+} and Ca^{2+}.** Stationary-phase cultures are more susceptible to this effect [72, 73]. **Lf has been reported to interact with the cell membrane of *Giardia* trophozoites, causing loss of permeability, vacuolization and damage to the plasmalemma, endomembrane and cytoskeleton** [72, 73].

Owing to the capacity of Lf to interact with a wide variety of proteins and macromolecules, it has been demonstrated that **its microbicidal action increases in synergism with other components of the immune system, such as immunoglobulin A (IgA) and lysozyme** [74, 75]. The activity may be due in part to two different mechanisms of action:

1) by its cationic nature, which gives the protein the capacity to bind Fe, cell surfaces and anionic molecules [76, 77, 78] and

2) by its serine-protease activity, immunomodulatory and immunostimulatory activity that, together with the mechanisms of the other components, may be responsible for the synergistic effect. In addition, **the synergistic effect has been proposed to originate from the interaction of Lf and its peptides with the membranes of bacterial pathogens, causing the bacteria to remain more susceptible to the effects of lysozyme and IgA** [79, 80].

5. Conclusions and perspectives

Several studies have shown that the administration of Fe to nursing mothers does not interfere with Lf saturation in their milk. In the first days of breastfeeding, it appears that Lf can exert a protective role for itself in the mammary gland. Recent studies have shown that children breastfed with maternal milk have higher concentrations of IgA than children breastfed with artificial formulas, suggesting that Lf stimulates a part of the immune response [81, 82]. Although there have not been detailed clinical assays, these observations support the effects of Lf found *in vitro*. However, it is necessary to know the details of effects to understand when to use them and how and in what ways they can help the host [37, 83].

In vitro assays have shown the potential for Lf as a broad-spectrum antimicrobial, including use with clinically isolated antibiotic-resistant bacteria, against which it presents the same effects as observed in the reference strains used in laboratory research. For all of these results, it is necessary to perform studies in patients to corroborate the effects described in this chapter. Until that time, the clinical assays developed thus far have shown that Lf has great potential as a nutraceutical and antimicrobial agent.

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Chapter 15

Fe and related proteins in *Toxoplasma gondii*

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Abstract

1. Introduction

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6. Tissue encystment

7. Treatment

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Abstract

Toxoplasmosis is an emerging oportunistic disease produced by the intracellular obligate parasite *Toxoplasma gondii* that is worldwide distributed. This infection affects severely to immunosupressed individuals as those infected by VIH-AIDS producing encephalitis and death. In pregnant women toxoplasmosis is related to newborn malformations and abortion.

To date, there are no antiparasitic drugs able to eliminate all the parasite forms in the infected host or a vaccine for human use. Combination of sulfadiazine and pyrimethamine is the most commonly used pharmacological treatment. Due to the limited effect of such drugs on the cyst form of the parasite, numerous experimental approaches have been achieved in order to design new compounds based in cell and molecular properties of the parasite.

Iron (Fe) is an essential metal for most living beings involved in different cell processes. It is obtained from food and absorbed through intestine. In *T. gondii*, Fe is taken from the invaded host cell with the participation of high affinity parasite molecules that compete with molecules from the host. This is an essential event in virulence and intracellular parasite proliferation.

Some Fe related proteins such as Ferritin and Lactoferrin diminish invasivity and inhibit intracellular replication of tachyzoites while in infected mice this cation increases their survival. At the same time, Fe related proteins activate host gut immune system against tissue cysts and bradyzoites. Nevertheless, due to the limited information about the role of iron in *Toxoplasma*, the study of Fe and related proteins for their application with therapeutic approaches could represent an alternative in the search of new pharmacological strategies against this dangerous pathogen.

Key words: ferritin, iron, lactoferrin, *Toxoplasma gondii*.

1. Introduction

Toxoplasma gondii is an important **oportunistic intracellular obligate pathogen** that belongs to the **phylum *Apicomplexa*,** which includes numerous parasite genus of medical and veterinary relevance such as ***Plasmodium, Eimeria*** and ***Cryptosporidium*** [1, 2, 3]. *T. gondii* invades a large variety of mammal and bird hosts with a broad geographic distribution. About 25% of world human population is infected by *Toxoplasma* [4]. Due to the high incidence of toxoplasmosis in AIDS patients, in recent years the study of the physiopathological properties of *T. gondii* has increased [5].

Toxoplasmosis can cause severe damage in humans resulting in death, mainly in immunosupressed patients as well as in fetuses and newborns. The most frequent alterations include **chorioretinitis** with consecutive **loss of vision and damage in central nervous system, lungs, heart and in extreme cases abortion and death** of the immunosupresed infected adult [6,7,8]. In healthy immunocompetent individuals, infection is transient and asymptomatic. In Mexico, a wide distribution of this parasitic disease has been reported with the highest prevalence of sera antibodies (up to 65%) at the coastal areas [9]. The success of *T. gondii* as intracellular pathogen is based on its high invasiveness and tissue dissemination, as well as in its location within tissue cysts in the form of **bradyzoites,** a form of low proliferation which remains active for all the life time of the infected host.

2. Life cycle of *T. gondii*

Life cycle involves two kinds of **hosts**: **definitive hosts**, which include felines, as the domestic cat in which sexual reproduction cycle takes place, and **intermediate hosts**, that include all warm blooded animals such as livestock cattle, sheep, pigs, etc; and humans in which the asexual reproduction occurs [4,10], (Figure 1).

The cycle begins when the cat eats infected prey whose tissues contain tissue cysts containing **bradyzoites (**a slow replication chronic form of *Toxoplasma***).** The bradyzoites are subsequently released into the intestine by digestive enzymes, beginning the invasion of intestinal enterocytes and their proliferation and differentiation into **macrogametes** (female cells) that are fertilized by the **microgametes** (male cells) resulting in the formation of a **zygote** [11,12] (Figure 1). **Zygote** transforms in an immature non-infective **oocyst that is released in cat feces** contaminating water supplies, soil and vegetables. **Under adequate temperature and humidity, oocysts mature into an infective oocyst containing 8 sporozoites** (Figure 1) [11, 13].

Humans become infected by ingestion of mature oocysts (containing sporozoites) or undercooked meat contaminated with tissue cysts (containing bradyzoites) (Figure 1). In both cases parasites are released and start **a rapid invasion of intestinal epithelium, once inside, they differentiate in tachyzoites, the fast and highly invasive form of *Toxoplasma* which replicates within a vacuole (PV) by endodiogeny,** an asexual process of cell division in which two daughter parasites are formed within a mother cell. The

saturation of intravacuolar space triggers the externalization of the tachyzoites with consecutive destruction of host cells and rapid invasion of neighboring cells [14].

Tachyzoites disseminate through the whole organism by **blood and lymphatic routes.** When host immune system becomes activated due to the presence of the parasite, **immune cells such as macrophages and lymphocytes respond with proliferation, activation and release of diverse cytokines** including gamma interferon (**IFN-γ**). Presence of IFN-γ induces tachyzoite differentiation into bradyzoites and modification of the host cell into tissue cysts giving place to chronic infections [13,15].

When infected individuals become **immunocompromised** by different reasons (VIH infection, immunosupresor treatments, cancer, etc), bradyzoites are reactivated and leave of tissue cysts to immediately invade neighboring cells and differentiate into tachyzoites. **Reactivation of a chronic infection into an active infection, involves parasite migration through the blood-brain barrier causing encephalitis and death** [4]. When infection occurs during pregnancy, parasites reach the placenta and infect the fetus. Damage in eye, brain or even abortion depend of the pregnancy period in which infection occurs [16, 17].

3. Structure of *T. gondii*

The most invasive and known form of *Toxoplasma* is the tachyzoite. It has a crescent shape with 8 µm of length and 2 µm of diameter [4]. Tachyzoite has a **trimembranal pellicle** that surrounds the entire parasite [18]. It has a nucleus, a single elongated mitochondria, endoplasmic reticulum and Golgi (Figure 2). At the apical end (the most pointed end) there is a retractile organelle known as **conoid** which plays an essential role during cell invasion [19]. *Toxoplasma* **cytoskeleton is formed by 22 subpellicular microtubules** and a cross-linked filament network rich in **actin** [20].

Toxoplasma has a non-photosynthetic plastid called **apicoplast**, which is **a multifunctional organelle involved in synthesis of lipids and isoprenoids,** both components necessary for the survival of the parasite [21].

Toxoplasma has three types of secretory organelles (**micronemes, rhoptries and dense granules**) that store proteins that are secreted sequentially during invasion and intracellular proliferation. [22]. **MIC** proteins stored in the micronemes are secreted during **gliding motility** and adhesion to the host cell membrane [23,24], **ROP** proteins stored in the rhoptries are secreted afterwards the apical end of the parasite is pushed down over the host cell membrane during the early stages of invasion [25,26] and **GRA** proteins stored in dense granules are exclusively secreted during proliferation and within the intravacular space of the PV [27] (Figure 2).

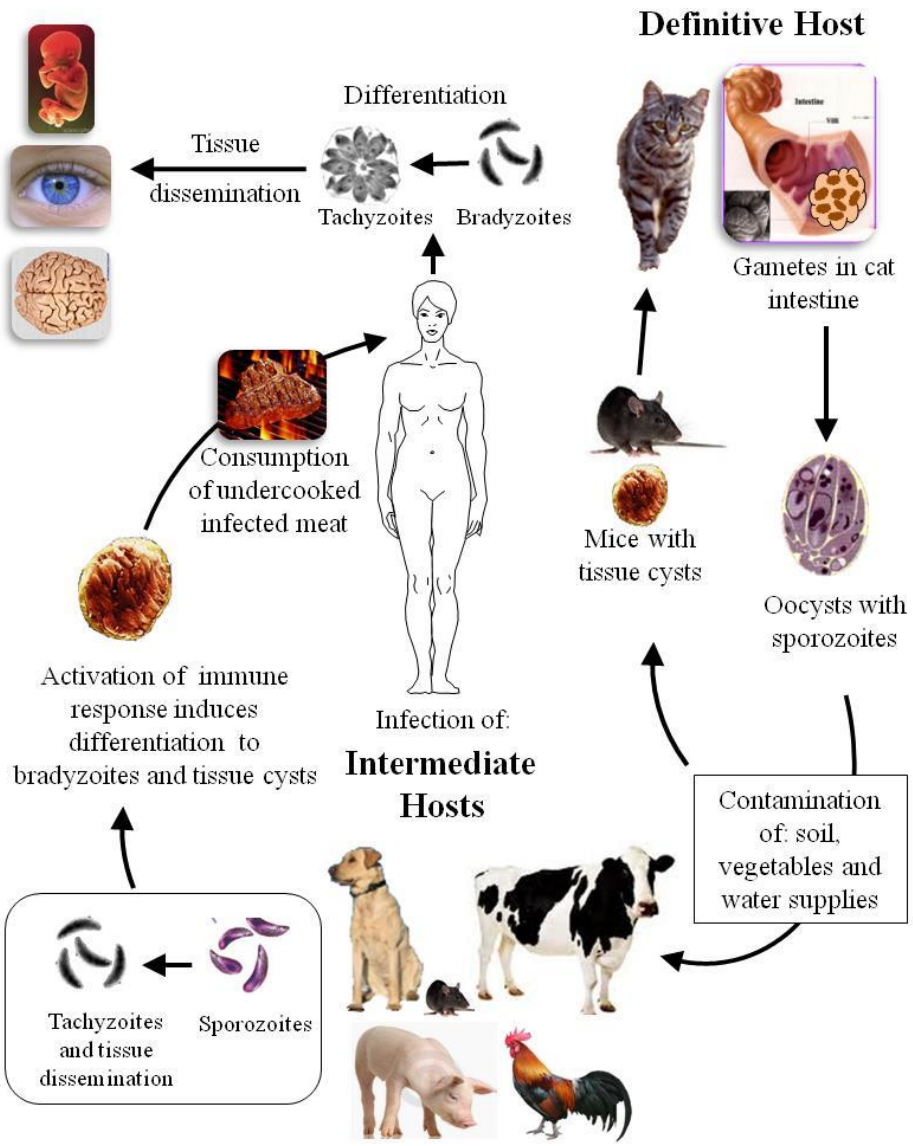

Figure 1. LIFE CYCLE OF *Toxoplasma gondii.* (R. Mondragón)

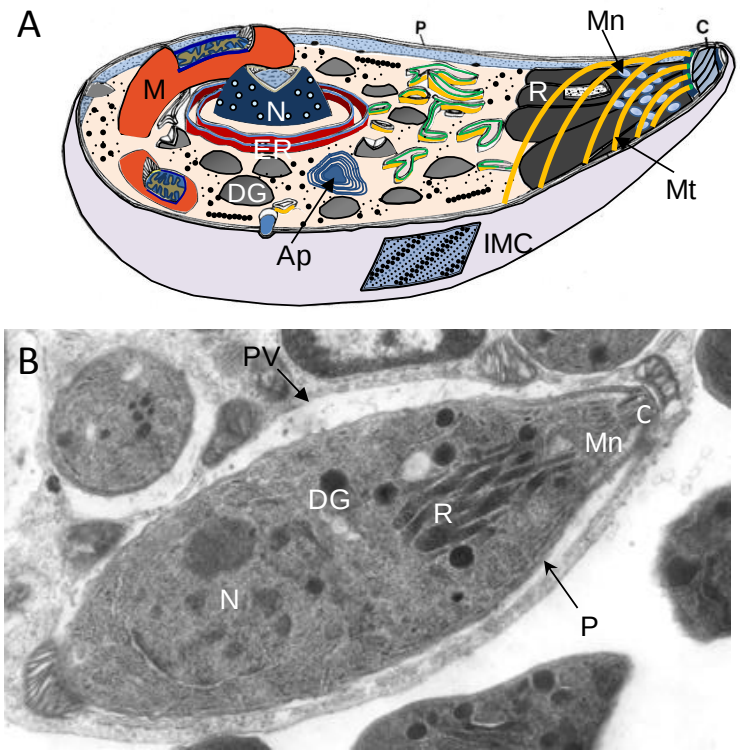

Figure 2. MORPHOLOGY OF *T. gondii* TACHYZOITE.

A. Diagram of a tachyzoite; B, EM micrograph of an intracellular tachyzoite within a peritoneal macrophage. (R. Mondragón**).**

Ap, apicoplast; DG, dense granules; IMC, intramembranal complex; M, mitochondria; Mn, micronemes; Mt, microtubules; N, nuclei; P, pellicle; PV, parasitophorous vacuole; R, rhoptries.

4. Cell invasion by *T. gondii*

Tachyzoite invades all types of cells through two mechanisms:

1. **Phagocytosis.** Involves internalization of the parasite by macrophages and neutrophils where these phagocytic cells are the effector cells [25]. The parasites are internalized into phagosomes that are subsequently modified to form a PV lacking of markers for lysosomal fusion, thereby enabling the survival of the tachyzoite.

2. **Active invasion.** Tachyzoites as well as the effector cells, display a set of events to invade host cells including: adhesion to the host cell membrane by MIC proteins, motility activation and conoid extrusion, secretion of rhoptries and formation of a 1 µm hole through which the parasite enters the host cell by twirling movements (Figure 3). While the parasite is entering the cell, a membranous vacuole is formed, giving place

to the PV. Once inside the PV, secretion of GRA proteins starts and iniciates the formation of the intravacuolar vesicle-tubular network [28].

Inside the PV, *Toxoplasma* initiates a constitutive secretion to the intravacuolar space of GRA 2, GRA 4, GRA 6 and GRA 9; All together form multiproteic complexes as well as the vesicle-tubular network [29, 30, 33, 34]. Intravacuolar location provides to the tachyzoites a protection against the lytic action of the enzymatic contents released after lysosomal fusion [33]. PV membrane becomes associated to organelles of the host cells such as mitochondria and endoplasmic reticulum (ER) [36, 37]. Intravacuolar tachyzoites divide by an asexual process known as endodiogeny, which is characterized by a synchronic division of two daughter cells within a mother cell [32]. Division time of *Toxoplasma* takes place in about 6 – 8 hours with an exponential proliferation in the following 48 hours within the PV [31].

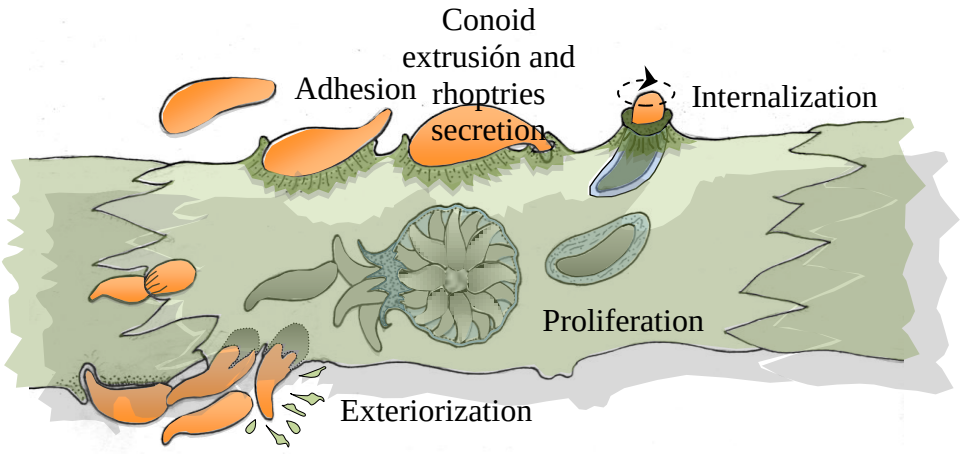

Figure 3. ACTIVE INVASION OF *Toxoplasma*.

Events include: Adhesion to the host cell membrane through MIC proteins, conoid extrusion followed by rhoptries secretion and formation of a hole in the host cell membrane followed by the internalization into a PV. Once inside the PV, tachyzoites proliferate by endodiogeny. By signals not yet identified, the tachyzoite is externalized with consecutive breaking of the host cell (R. Mondragón**).**

5. Tissue dissemination

An important feature of *T. gondii* infection is the parasite dissemination to all the tissues. **Tachyzoiye can cross all biological barriers such as intestine, blood-brain barrier, blood-eye barrier and placenta during the primo-infection or during the reactivation of the chronic disease** [37]. Migration through these biological barriers depends on parasite motility and probably takes place following a **paracellular route** in

order to reach blood circulation and distribute throughout all the tissues [38]. Recognition of immunoglobulin superfamily cell adhesion molecules (**ICAM**) of the host cell by *Toxoplasma* has been considered as an important event during the trans-epithelial migration [38].

6. Tissue encystment

As result of the infection with *Toxoplasma*, host immune system is activated with production of **IFN-γ** as well as other cytokines as a molecular strategy to activate effector immune cells against the parasite [39]. Recent reports have indicated that the presence of **IFN–γ activates** a mechanism not well determined, **a differentiation process of the intracellular tachyzoites into bradyzoites with consecutive modification of the host cell into a tissue cyst**.

Tissue cysts and bradyzoite identification has been possible by the recognition of specific carbohydrates such as **α-N-acetyl-galactosamine** with a lectin obtained from *Dolichos biflorus* [40, 41, 42]. Encystation requires a period between 4-6 days post-infection. Composition and structure of the cysts are the main attributes that provide *Toxoplasma* the resistence against host defense mechanisms and parasiticide drugs.

7. Treatment

To date, there are no efficient treatments available to eliminate all forms of *Toxoplasma* in the host. Bradyzoites and tissue cysts are infective forms that cannot be easily destroyed by conventional anti-toxoplasmic treatments. Compounds that have been used in toxoplasmosis treatment include: **sulfonamides, clyndamicin, macrolides and hydroxynaphthoquinones.** All these agents show clear effects against viability of extracellular tachyzoites, however they have limited or null actions on intracellular parasites located within PVs or on tissue cysts [43, 44].

The most used treatment in toxoplasmosis consists in a combination of pyrimethamine and sulfonamide. A mixture of **spiramycine** with the mentioned drug combination, represents the choice treatment for congenital toxoplasmosis during pregnancy [45]. Recent pharmacological approaches have been focused to the molecular design of drugs directed against specific parasitic metabolic pathways such as those present in the apicoplast, a vital organelle generated from an endosimbiotic process, and that is involved in synthesis of certain vital lipids for the parasite. **Drugs that have been recognized to affect apicoplast metabolism include fluoroquinones, triclosan, chloramphenicol and macrolides** [46].

8. Role of iron and accesory proteins in the cell biology of *T. gondii* and Apicomplexans

In spite of the fact that iron (Fe) is an indispensable element for survival of all mammalian cells, study of importance of Fe in Apicomplexans such as *Toxoplasma* as well as in its relationship with the host cell are fields that have been partially explored.

Some authors have suggested that Fe and some related proteins, have essential roles during proliferation and survival of intracellular Apicomplexan parasites of high relevance in human beings and animals. **Apicomplexans in which Fe has been demonstrated to be a relevant cation are: *Plasmodium ssp., Babesia ssp., Cryptosporidium spp., Isospora belli, Cyclospora cayetanesis* and *Toxoplasma*. Capture of Fe from the host cell by these parasites represents an essential event in their virulence** [47].

Ferritin has been suggested as an important supply source of Fe during the growth of pathogens. *Toxoplasma* takes Fe from the host cell through the **expression of proteins with high affinity for the cation, thus competing with the typical carriers of Fe in the body** [47, 48].

Fe has been considered as an important and necessary component in the intracellular proliferation of *T. gondii*. Capture of Fe from the host cell is a key process that *Toxoplasma* has to regulate in order to secure a constant provision of such cation at the intravacuolar level. The parasite increases the expression of **iron regulatory proteins (IRPs)** which apparently function by stabilizing the mRNA corresponding to the **Transferrin receptor (TfR)** of the host cells, **thereby increasing the expression level of Transferrin at the membrane of the invaded cells.** This condition generates in the host cell a great inward flow of Fe, which is then taken by intracellular tachyzoites for their proliferation [49]. This **positive regulation** seems to be mediated by soluble factors secreted by *Toxoplasma* not yet identified [49].

The main secretory organelles that are discharged when tachyzoites have an intracellular location are the dense granules, which contents (GRA proteins) become inserted within the PV membrane and contribute also in the organization of the intravacuolar vesicular-tubular network [29]. **The particular membrane localization of some GRA proteins has suggested a feasible participation in the regulation of Fe incorporation from the infected host cell,** although to date the precise proteins involved have not been identified.

Due to the increasing importance of the role of Fe in intracellular proliferation of *Toxoplasma*, various strategies have been designed to **limit parasite development through the use of Fe chelating agents such as desferrioxamine** [50, 51]. In studies of *Toxoplasma* invasion and proliferation in primary cultures of rat enterocytes (cells that form part of the intestine epithelium), exposure to desferrioxamine **inhibited intracellular replication** of tachyzoites. This effect was reverted through the addition of exogenous **holo-Transferrin** or **ferrous sulfate** as sources of Fe, condition that allowed a successful intracellular replication of the parasite and demonstrated the importance of Fe in the intracellular development of this parasite [51].

Besides the participation of **macrophages and neutrophils** against *Toxoplasma*, it has been proposed that **fibroblasts and endothelial and intestinal cells** might help in the protection against this pathogen, probably through a mechanism that involves the incorporation of Fe from the plasma, limiting as consequence the availability of Fe resources to the parasite [51]. An alternative strategy has suggested the participation of exogenous **IFN-**γ in the inhibition of intracellular tachyzoites replication, a phenomenon observed in a dose-dependent in primary cultures of rat enterocytes [51]. Interestingly, the exogenous addition of **ferrous sulfate** and **holoTf** neutralizes entirely the effect of **IFN-**γ on the enterocytes. Although the precise molecular events activated in enterocytes by exposure to **IFN-**γ is not clear, it has been suggested that IFN-γ inhibits the tachyzoites replication by a mechanism that involves the limitation of available intracellular Fe [51].

In addition, incubation of HT-29 and Caco-2 cells (both intestinal phenotype strains) with IFN-γ before infection with ***Cryptosporidium parvum***, an Apicomplexan pathogen that infects specifically enterocytes, induced resistance of these cells to the infection, suggesting the existence of resistance mechanisms similar to that described for *Toxoplasma* [52].

Lactoferrin (Lf), a protein closely related with **Transferrin**, is a 80 kDa Fe carrier protein (Figure 4). It is formed by simple glycoprotein chains which affinity for Fe is slightly higher than Tf. It has a Fe retention in lower pH values and can bind **heparine** through a pseudo-specific way**.** As the name indicates, Lf was originally isolated from milk, where it is found in high concentrations and also is expressed during embryo development [53].

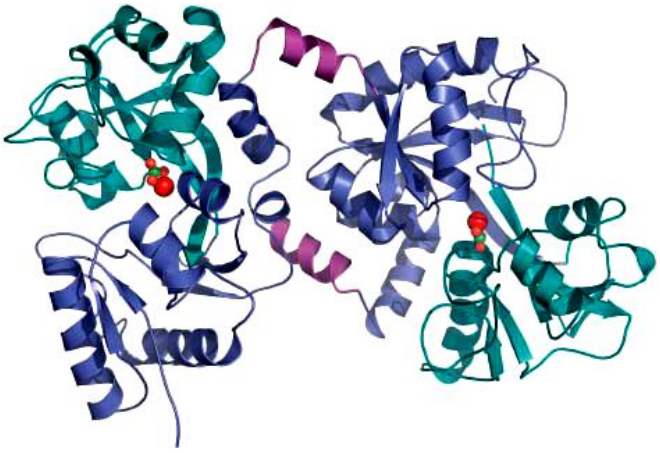

Figure 4. RIBBON REPRESENTATION OF THE CHARACTERISTIC POLYPEPTIDE FOLD FOUND FOR HUMAN LACTOFERRIN AND ALL OTHER MEMBERS OF THE TRANSFERRIN FAMILY.

The N-lobe is on the left, with its bound Fe^{3+} and CO_3^{2-} ions (shown as spherical atoms) in the cleft between the two domains, N1 (dark blue) and N2 (teal). (Modified from Baker EN & Baker HM. *Cell. Mol. Life Sci.* 62 (2005) 2531–2539)

In mammals, synthesis of Lf takes place in the **acinar glands** and it is **secreted into mucosae.** It is also synthesized and **secreted by neutrophils** in infection sites and by human kidney cells [54]. Lactoferrin, as a quelant molecule for Fe^{3+} has antimicrobial properties, which affects the development and growth of some virus, bacteria and protozoa through the trapping of Fe [55] or by a direct penetration of cell membranes [56]. In previous studies Lf has been suggested as an **anti-parasitic** molecule in some parasite genus such as ***Pneumocystis carinii, Entamoeba histolytica, Babesia spp, Plasmodium spp*** **and** ***T. gondii*** [57].

Previous studies have shown that *T. gondii* tachyzoites of the BK strain, bind to human holoLf but not to holoTf or apoTf; therefore, the presence of **specific membrane receptors of *Toxoplasma* to the Fe carrier proteins from the host cell** has been suggested [58]. Studies with fluorescency microscopy using biotin-avidin, showed the binding of Lf and ovoTf to the surface of *Toxoplasma* and by Western-blot in tachyzoites whole extracts, it was determined the presence of a 42-kDa protein able to specifically bind Fe proteins carriers, suggesting that **this molecule could correspond to the membrane receptor** [59].

In further studies, it was determined that both, the **lack or excess of Fe** in the culture medium, produced an inhibition of intracellular proliferation of tachyzoites of the RH strain, but the mechanism by which this phenomenon occurred is unknown. [60]. Based on the amount of Fe present in the Lf, it has been shown that **Lf binds to the negatively charged membrane of tachyzoites as a cationic compound,** a feature that allows its detection by EM as an electron-dense precipitate deposited on the outside of the *Toxoplasma* pellicle [61]. The binding of Lf to the surface tachyzoite, does not affect the invasive capacity of the parasite in leukocytes, macrophages and embryonic cells *in vitro* [62].

Lactoferricin (Lfcin), a peptide composed by 25 amino acids formed by pepsin digestion of Lf has harmful effects on parasites. Exposure of sporozoites and tachyzoites of *Eimeria stiedai* and *T. gondii*, respectively, to lactoferricin, induces a **decrease in their invasive capability** in embryonic rat cells. Similarly, mice previously treated with Lfcin, showed a higher survival index after intraperitonal inoculation of *Toxoplasma* tachyzoites in contrast with those untreated mice [62]. It has been proposed that the effect of Lfcin in these cases might be explained in two ways, one considers a direct masking action of Fe by the peptide and the other through a direct harmful action of Lfcin on the parasite membrane.

Effect of **Lfcin (0.5-5.0 mg/ml)** has been also evaluated in brain rat cysts obtained from the Berverley strain of *T. gondii*. Oral administration of Lfcin in infected animals showed a protector function dependent on the doses. From these results, it was suggested that **Lfcin could activate the host immune system** and as consequence facilitate the destruction of parasites and cysts. In oral infection in mice, Lfcin could have at the intestinal tract a direct harmful effect on tissue cysts and bradyzoites by an initial binding to the parasite membrane, destruction of the pellicle and decrease of the infective capability [62]. **Lactoferricin represents a promising antimicrobial agent** with applications in prophylaxis and therapeutic treatment.

Frataxin is a highly conserved protein that participates in the regulation of iron homeostasis in prokaryotes and eukaryotes (Figure 5) [63]. In eukaryotes is located in the mitochondria and has been recently shown to perform the dual function of making Fe^{2+} bioavailable and detoxifying surplus iron. Deficiency of this protein in humans causes a degenerative cardio-neurological disorder known as **Friederich's ataxia** [63]. **Frataxin** seems to be involved in **assembly of Fe-S clusters (ISCs)** that are closely related to the mitochondrial metabolism [64]. It has been proposed to act as either an iron chaperone or an iron storage protein

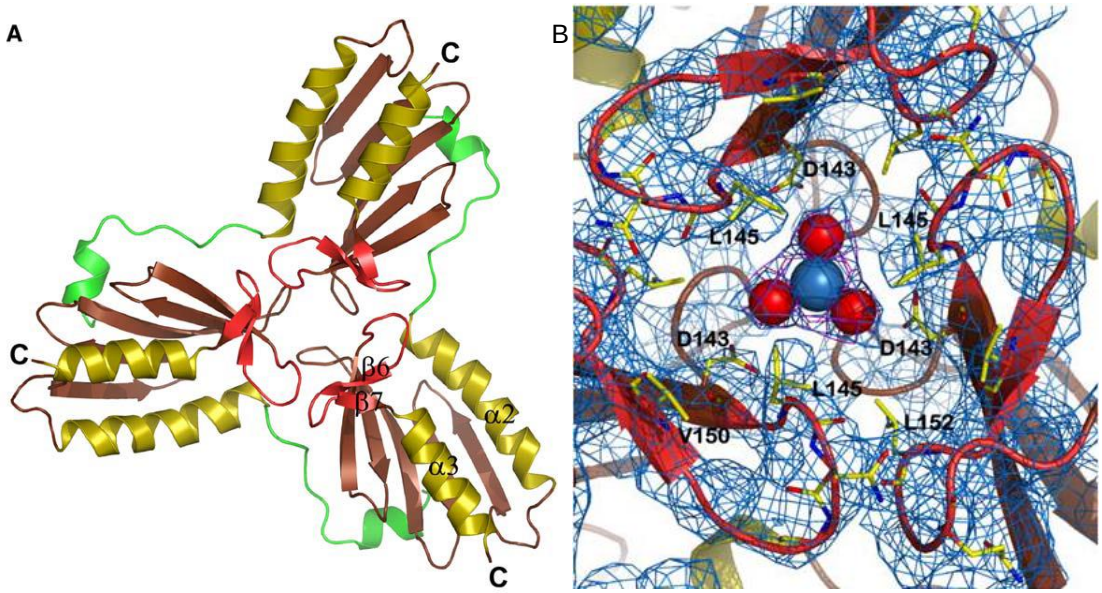

Figure 5. STRUCTURE OF THE YEAST FRATAXIN TRIMER.

(A) Suggested structure of the trimer in ribbon representation with the exposed helices α2 and α3 (shown in yellow). Loops α and β strands are shown in brown or red. (B) Top view of the channel with bound iron. Bound iron shown as a blue sphere is located about 6 A° from the entrance to the channel,

as defined by the side chain of L145. The red spheres correspond to solvent molecules. (Modified from Karlberg T, *et al*. *Structure, 2006,* 14: 1535-1546)

Fe-S clusters are involved in electron transportation, enzymatic catalysis and regulation of gene expression. In yeast and other eukaryotes, mitochondrion is the organelle in where these molecules are synthesized. **Detection of ISCs in the apicoplast of Apicomplexans, has suggested that they could be synthesized in such organelle together with vital enzymatic complexes involved in synthesis of lipids and folic acid in *Toxoplasma*;** It is possible that apicoplast contains the necessary molecular elements for the synthesis of their own ISCs [65, 66].

A **Ferredoxine-NADP reductase** from *T. gondii* and a **Ferredoxine cluster** from *P. falciparum* were recently cloned [67]. Both proteins constitute part of the redox systems extensively characterized in photosynthetic organisms as the responsible molecules in the following transference of electron:

$$2Fd_{red} + NADP^{+} + H^{+} \longleftrightarrow 2Fd_{ox} + NADPH$$

In the non-photosynthetic plastids (and very probable in the apicoplast), the transference of electrons may be given in the inverse sense from NADPH to $ptFd_{red}$, working likely as a reducing condition in diverse metabolic functions.

8.1 Ferritin as cationic marker

The negative electric charge of the tissue cyst surface of *T. gondii* is a property produced by the presence of certain phospholipids, which was demonstrated through the use of the cationic Ferritin [68]. **Cationic Ferritin** is a tool that has been extensively used to evaluate the participation of secondary lysosomes in phago-lysosomal processes during phagocytosis of *Toxoplasma* tachyzoites [69].

A study to determine the electric charge of the tachyzoites surface by the binding of colloidal gold and cathionic Ferritin particles in infected host cells, demonstrated that ***Toxoplasma* presents positive and negative charges** [61]. Ferritin particles are deposited at the limit of the PV membrane but not in intravacuolar tachyzoites, which indicates a differential distribution of charges and molecules in the parasite. The biological meaning of this characteristic is unknown [70].

9. Conclusions

Nevertheless the few reports about the role of Fe in *Toxoplasma*, the available data indicate the great importance that Fe has in its pathogenicity. Participation of Fe in the intracellular development of *T. gondii* is vital for the parasite particularly during intracellular replication. Expression of **IPRs** in the tachyzoites, facilitates capture and efficient transportation of Fe as well as an adequate use and storage of the metal by the parasite.

The role of **GRA** proteins in the capture of Fe capture is not clear to date, but it could implicate the activation of IPRs by the parasite. Therefore, the study of IPRs during the host-parasite interaction should be considered as an important issue in the search of new targets for the design of new therapeutic approaches. On the other hand, by the use of chelating agents, ferrous sulfate, holoTransferrin and IFN-γ exogen, is clear that the bioavailability to Fe regulated by the own organism could represent a host strategy to limit the intracelular growth of the parasites.

At present, most treatments used in toxoplasmosis are focused to control the acute state of the infection, while the chronic stage remains untreated because the limited access of the drugs to the intravacuolar space or to the interior of the tissue cysts. Inhibitory effects of a molecule related to the iron metabolism such as **Lactoferricin** in growth, proliferation, and viability of parasites (tachyzoites and bradyzoites) generates great expectations in the search of new anti-*Toxoplasma* therapeutic strategies.

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Chapter 16

The Iron effect in the biology of *Trichomonas vaginalis* and *Tritrichomonas foetus*

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Abstract

Both *Trichomonas vaginalis* and *Tritrichomonas foetus*, human and bovine sexually-transmitted anaerobic protozoa, respectively, have high-iron requirements and a great dependence on this cation. Iron is essential for their growth, metabolism, multiplication, and for virulence regulation by mechanisms that are not yet well identified and characterized, especially in *T. foetus.*

Due to the high-iron requirement, these protozoa have developed several mechanisms and specializations to acquire iron from different sources available in the vaginal microenvironment during infection. For example, the iron sources for *T. vaginalis* are Hemoglobin, Lactoferrin, Hemin, Cytochrome *c*, epithelial cells, and erythrocytes; while *T. foetus* uses Lactoferrin, Transferrin, Hemin, iron chelators, and xenosiderophores as iron sources.

In *T. vaginalis* we are starting to understand the mechanisms through which gene expression is regulated by iron, both at the transcriptional level (mediated by iron-responsive promoters and DNA-protein interactions) and at the post-transcriptional level (mediated by RNA-protein interactions through an IRE/IRP-like system), similar to the one involved in the maintenance of mammalian intracellular iron homeostasis. These mechanisms will help to understand the way in which this parasite can differentially modulate the expression of some of the molecules involved in its virulence as an adaptative response to constant fluctuations in the iron concentrations throughout the menstrual cycle. This differential expression prepares and helps *T. vaginalis* to remain for a long time in the host, causing a chronic infection with severe consequences.

Due to the evolutionary relationship between *T. vaginalis* and *T. foetus*, it is possible that these two protozoa could use similar mechanisms of iron-regulated gene expression.

Key words: adherence; adhesins; complement resistance; cysteine proteinases; cytotoxicity; hemolysis; hairpin structures; hydrogenosomes; iron; iron-acquisition mechanisms; iron-responsive promoter; IRP-like proteins; iron-responsive elements (IRE); lactoferrin; post-transcriptional regulation by iron; RNA-protein interactions; siderophores; transferrin; *Trichomonas vaginalis*; *Tritrichomonas foetus*; transcriptional regulation by iron; virulence properties; virulence factors.

1. *Trichomonas vaginalis* and iron

1.1. Introduction

Trichomonas vaginalis is a **flagellated parasite protozoan** responsible for **human trichomonosis,** one of the most common **non-viral sexually transmitted** infections worldwide. Nowadays, it is a public health problem at the pandemic level.

Besides **vaginitis, urethritis,** and **prostatitis,** trichomonosis main complications are **endometritis**, **infertility**, and a high risk in pregnancy due to rupture of the amniotic membranes. It is also considered a **risk factor for cervical and prostatic cancer** and **increase of susceptibility to infection and transmission of the human immunodeficiency virus** (**HIV)** [3,96].

In the human urogenital tract, *T. vaginalis* is exposed to adverse conditions such as: acidic pH, lactobacilli, cyclic hormonal changes, epithelial desquamation, lack of nutrients, menstrual flow, and **fluctuations in iron concentration throughout the menstrual cycle**. This protozoan is capable of crossing the vaginal mucus, adhering to the epithelium, multiplying, and colonizing the urogenital tract, hence causing a chronic infection in humans [3,20,69].

Thus, to infect humans and cause a chronic infection, *T. vaginalis* requires a **great adaptative capacity,** as well as using multiple resources such as:

1) **Nutrient acquisition through specific receptors**;

2) **Mucin interaction and degradation,** followed by **adherence to the vaginal and cervical epithelium** through **adhesins** that have homology to metabolic enzymes;

3) **Multiple strategies for host immune evasion**: a) **covering with host protein,** b) **protein release** into secretions to distract the immune response, c) **cysteine proteinases (CPs) secretion** that degrade all types of immunoglobulins as well as the complement, d) **molecular mimicry**;

4) **Antigenic variation** or the **alternate expression of at least two surface antigen groups;** and

5) The **coordinated and alternated expression of virulence genes** in response to host environmental factors.

That the parasite survives for a long time in the adverse and variable vaginal microenvironment is evidence of the **highly evolved nature of this protist**, which has the capacity to adapt and multiply in the vagina, despite the variable conditions during the menstrual cycle [3,6,9,10,20,48,67,69,98].

Both *T. vaginalis* **adherence and cytotoxicity** to the host cells **depend on virulence factors**, such as **adhesins, CPs**, **laminin- and fibronectin-binding proteins**, **integrins**, **cell-detaching factors**, **pore-forming proteins, and glycosidases**, among others. The way in which t**richomonads receive and respond to environmental signals** by stimulating or repressing the release of certain molecules, and/or the activity of some surface enzymes, or the expression of some genes, is the way wherein the pathogenicity of this protozoan is affected [2,3,16,17,18,20,65,66,67,68,69]. **Iron is one of the key environmental factors that greatly affect *T. vaginalis.***

***T. vaginalis* needs a high-iron concentration (250-300 μM)** for an adequate growth and metabolism. Iron is essential for *T. vaginalis* multiplication and metabolism [39]. In the vagina, the parasite is subjected to cyclic variations of iron concentration during the menstrual cycle. Multiple studies have shown that these iron variations either positively or negatively modulate the expression of several molecules, affecting the virulence properties in which they are involved with [7,9,10,13,15,29,30,31,32,33,38,39,48,49, 57, 62,63,65,66,68,69,70,71,72,73,74,82,83,84,89,90,91,92,93,95,102,104,105,109,117,118,119].

1.2. Iron effect in *T. vaginalis* growth, metabolism, and virulence.

Iron is essential for *T. vaginalis*, since when parasites are grown in low-iron concentrations the protein synthesis rate decreases up to 80%, the cell density reduces at least 3 times, its generation time increases 2.5 times, giving a generation time of about 10-24 h [66]. **The lack of iron causes drastic morphologic changes**; **ovoid or amoeboid parasites become rounded, the axostyle and flagella internalize, appearing pseudocyst morphology** [31]. There is also an **increase in the Lactoferrin (Lf) binding capability, possibly by an increase in the Lactoferrin receptor (LfR) expression** [66,67].

In the absence of iron, at least 45 proteins are differentially regulated: some decrease or are absent [enzymes involved in energy metabolism, proteolysis and **hydrogenosomal** iron-sulfur proteins (**Fe-S**)]**; while others are overexpressed** (six **actin** isoforms, **phosphoenolpyruvate carboxykinase, one putative lactate dehydrogenase, and putative adenosin triphosphatase**) [32].

Hence, **iron is a key element that regulates cell morphology and protein expression** [32] *in vitro* and *in vivo*, given that **at least 19 differential bands of iron-modulated immunogens are detected in sera of trichomonosis patients** [66]. The differential gene expression regulated by iron of the **LfR,** metabolic enzymes, immunogens, and virulence factors shows the relevance of this cation in growth, metabolism, and virulence of *T. vaginalis* for maintenance of a chronic infection in humans.

These data are consistent with the behavior of *T. vaginalis* in the animal mouse model. O**nly parasites grown in iron-rich conditions produce a subcutaneous abscess.** In *in vitro* assays, the lack of iron decreases adherence and cytotoxicity levels toward HeLa cells monolayers [102]. However, these data are controversial because our studies show that *T. vaginalis* parasites grown in the absence of iron are highly cytotoxic over HeLa cells monolayers, which is related to an increase in the expression and proteolytic

activity of a 65-kDa proteinase, **TvCP65** [12,13], and two 30-kDa CPs recently described, **TvCP39** and **TvCP12**, also down-regulated by iron. These differences could be explained because these groups work with different trichomonad isolates. In addition to, new data show that **the lack of iron increases the induction of apoptosis in epithelial cells,** due to an increase in the proteolytic activity of some secreted proteinases identified by mass spectrometry as CP2, CP3, CP4, and CPT [63].

Iron plays a key role in *T. vaginalis* cytopathogenicity [102] and it has a **pleiotropic effect** over its virulence properties. Iron has a positive effect over adherence [68], complement resistance [9], and its capacity to bind fibronectin [2,3,29]; however, it has a negative effect over cytotoxicity [13] and induction of apoptosis in epithelial cells [63]. The lack of iron favors these properties. These data suggests that *T. vaginalis* may have **sensors to detect the iron level and the ability to respond to iron variations, as well as mechanisms to differentially regulate the expression of its virulence.**

1.3. Adherence and cytotoxicity are modulated by iron concentrations

1.3.1. Adherence

T. vaginalis adherence to the vaginal epithelium is one of the first events in the development of its pathogenicity; **it is a multifactorial process in which host (receptors), parasite (adhesins and proteinases), and vaginal microenviromental factors (pH, iron, hormones, redox potential, polyamines, among others) are involved** [3,4,5,8,16,17,18,66,68].

At least five surface proteins have been identified as trichomonad adhesins (AP120, AP65, AP51, AP33, and AP23, according to their molecular weight) [5,18,84] (Figure 1A) and **at least two CPs**, TvCP30 [16,17,82] (Figure 1B), as well as TvCP62 [47] are necessary for *T. vaginalis* adherence to cervical (HeLa) and vaginal epithelial cells (VEC). These two groups of proteins are some of the **trichomonad virulence factors** that participate in cytoadherence and are differentially modulated by iron.

Adhesins are up-regulated by iron at the transcription and translation levels [8,19,68,84], **and by contact to the host cells** [38,61]. Iron also participates in their differential **cellular compartmentalization** [38,84]. Three of four of these adhesins (AP65, AP51, and AP33) (Figure 1A) show **high homology to host and parasite metabolic enzymes** (to **Malic enzyme** –AP65- and to **Succinyl-coenzyme A synthetase α and β subunits** –AP33 and AP51, respectively); the exception is AP120 with homology to a parasite metabolic enzyme, the **Pyruvate:Ferredoxin Oxydoreductase (PFO),** which is absent in humans [3,6,18,19,68,83,84]. **All these metabolic enzymes are located in hydrogenosomes, but as adhesins they are also located on the parasite surface.**

These molecules become examples of the ***T. vaginalis* functional diversity of some proteins** [6,54]. The multifunctionality of the **AP65 and AP33** adhesins has been recently demonstrated by **different**

approaches: antisense RNA gene silencing [87,88] **and heterologous transfection of the *ap65* gene to *Tritrichomonas foetus*** [60].

1.3.2. Cytotoxicity

T. vaginalis cytotoxicity to epithelial cells is also a multifactorial phenomenon wherein **porins, toxins, cell detachment factors, phospholipases** [3,20,96], **and CPs such as TvCP65** [12] **and TvCP39** [48,49] (Figure 1B) participate. The CPs are localized on the parasite surface, **have affinity for the surface of vaginal and cervical cells, and degrade several natural substrates: fibronectin, immunoglobulins, and collagen I and IV.** Both expression and proteolytic activity of these proteinases are down-regulated by iron [13,49].

1.4. Proteins and functions up-regulated by iron:

***T. vaginalis* is capable of binding fibronectin in a specific receptor-mediated manner** and this is positively influenced by iron, calcium, and possibly by the participation of phosphatases [29].

Iron is also required for the enzymatic activity of ecto-ATPases and ecto-phosphatases [33], **the expression of a 44 kDa surface protein (TV44) that binds Immunoglobulin A (IgA)** [89]**, and a Triacylglycerol lipase (TVLip) at the transcript level,** similar to lipases from the EC3.1.1.3 group [26]. **The Nucleoside Triphosphate Diphosphohydrolase 1 (NTPDase 1 that hydrolyses extracellular ATP to ADP) and ecto-5'-Nucleotidase (that hydrolyses AMP)** are also positively regulated by iron at the protein and enzymatic activity levels [115].

Some CPs are also positively regulated by iron such as **TvCP4 and TvLEGU-1** at the transcript and protein levels [104,105], as well as their localization on the surface of *T. vaginalis* [24]. Besides, there is an **increase in the proteolytic activity of some parasite CPs that degrade the C3b complement component** [9]**, favoring the trichomonad complement resistance and suggesting** that ***in vivo T. vaginalis* might become more resistant to complement in the presence of menstrual blood.**

Iron also favors the modification of the intercellular tight junctions of Caco-2 cell monolayers after parasite adherence; transepithelial resistance decreases and the proteins distribution pattern from complex junctions such **as E-cadherin, occluidin, and ZO-1** changes, enhancing the intercellular spaces [31]. However, the parasite proteinases involved in these tight junction modifications have not been identified.

Adhesins expression is positively regulated by iron (Figure 1A**) and by contact with the host cell, at both transcription and protein levels** [19,38,61,68]. For example, AP65 is encoded by at least three different genes (*ap65-1*, *ap65-2*, and *ap65-3*) that show 88% to 96% identity, are **multicopy,** and have 38% **identity with the malic enzyme**. Even though all of them are positively regulated by iron, they have differences in their regulatory regions, especially at their 3'-UTR that could be related with the **differential degradation of AP65 transcripts** [6,8,19,90].

Even though there are at least 3 different genes for AP51 with homology to the β subunit of the succinyl-coenzyme A synthetase, only two of them are positively regulated by iron (*ap51-1* and *ap51-3*) [3,6,20,67]. Moreover, the expression of AP120 with homology to PFO A is only induced in the presence of iron [84]. Although there are at least seven *pfo*-like genes [25,83] in the *T. vaginalis* genome, only *pfo a* and *pfo b* are transcribed**; *pfo b* in a constitutive manner (with a slight increase in high iron) and *pfo a* is induced in iron-rich conditions** [83,84] **(**Table 1**).**

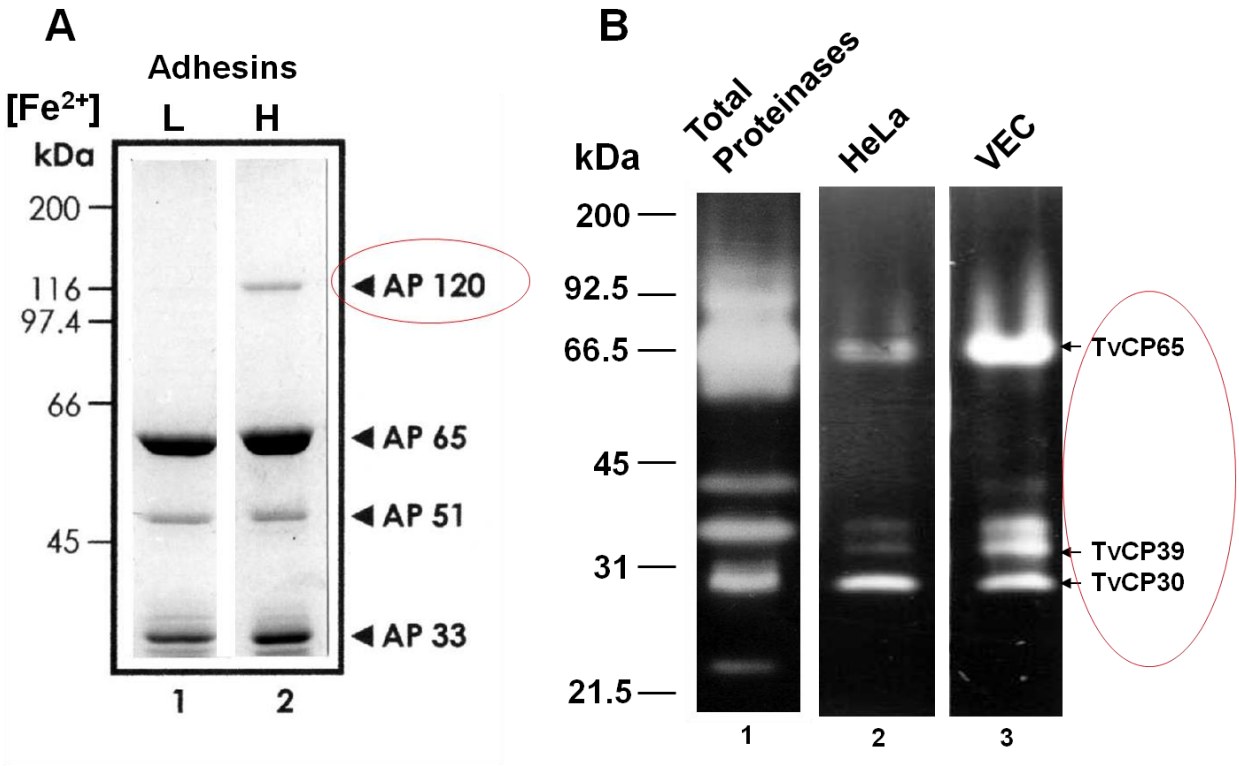

Figure 1. *Trichomonas vaginalis* PROTEINS WITH AFFINITY TO HeLa CELLS SURFACE CHARACTERIZED AS VIRULENCE FACTORS: ADHESINS (A) AND CYSTEINE-PROTEINASES (B).

A. Adhesin pattern from parasites grown in low (L, lane 1) and high iron concentrations (H, lane 2) obtained by a cell-binding assay and analyzed by electrophoresis in 7.5% polyacrylamide Coomassie blue-stained gels; kDa, molecular weight markers; arrowheads show the position of adhesins (AP) named according to their size as AP120, AP65, AP51, and AP33 [18,68,84**].**

B. Zymograms of proteinases from *T. vaginalis* extracts (lane 1) and cysteine proteinases (CPs) with affinity to the surface of HeLa (lane 2) or vaginal epithelial cells (VEC, lane 3) obtained after cell-binding assays. TvCP65 (CP65), TvCP39 (CP39), and TvCP30 (CP30), CPs of 65, 39, and 30 kDa characterized as virulence factors [12,17,48,49,82**].**

Table 1. POSITIVELY REGULATED PROTEINS IN IRON-RICH CONCENTRATIONS

Proteins	Participate in:	References
AP65, AP51, AP33, AP23, and AP120	Adherence and as Hemoglobin- and Heme-binding proteins.	[3,5,18,68,84]
Ferredoxin, Succinyl-CoA synthetase, Pyruvate: ferredoxin oxidoreductase (PFO)	Carbohydrate metabolism	[39,52,55,86]
TvLEGU-1 legumain-like cysteine proteinase	Adherence	[71,99,104]
TvCP4 cathepsin L-like cysteine proteinase.	ND[a]	[24,105]
Ecto-ATPases and ecto-phosphatases	Surface proteins	[33]
Triacylglycerol lipase (TVLip)	Similar to group EC3.1.1.3 lipases	[26]
Nucleoside triphosphate diphosphohydrolase 1 (NTPDase 1)	Extracellular ATP hydrolysis to ADP	[115]
Ecto-5'-nucleotidase	AMP hydrolysis	[115]
Cysteine-proteinases	C3b complement component degradation	[9]
Some proteinases	Tight junction alterations and reduction of transepithelial resistance	[31]

[a]ND, not determined

1.5. Proteins and functions down-regulated by iron:

Similar to molecules whose expression depends upon the iron presence, there are others that are only expressed in its absence. Usage of different **iron chelators** in the culture medium: **Deferoxamine (DFO)** [78] and **2-2'Dipyridyl** has been very useful to determine this [13,32,33,39,68].

Some **examples of molecules negatively regulated by iron are: LfR**, **a 136 kDa surface protein** [66], **P270 immunogen** that undergoes antigenic variation in isolates carrying the double-stranded RNA virus [10], **CPs involved in the contact-dependent cytotoxicity**, **TvCP65** and **TvCP39** [13,48,49] **(**Figure 2**).**

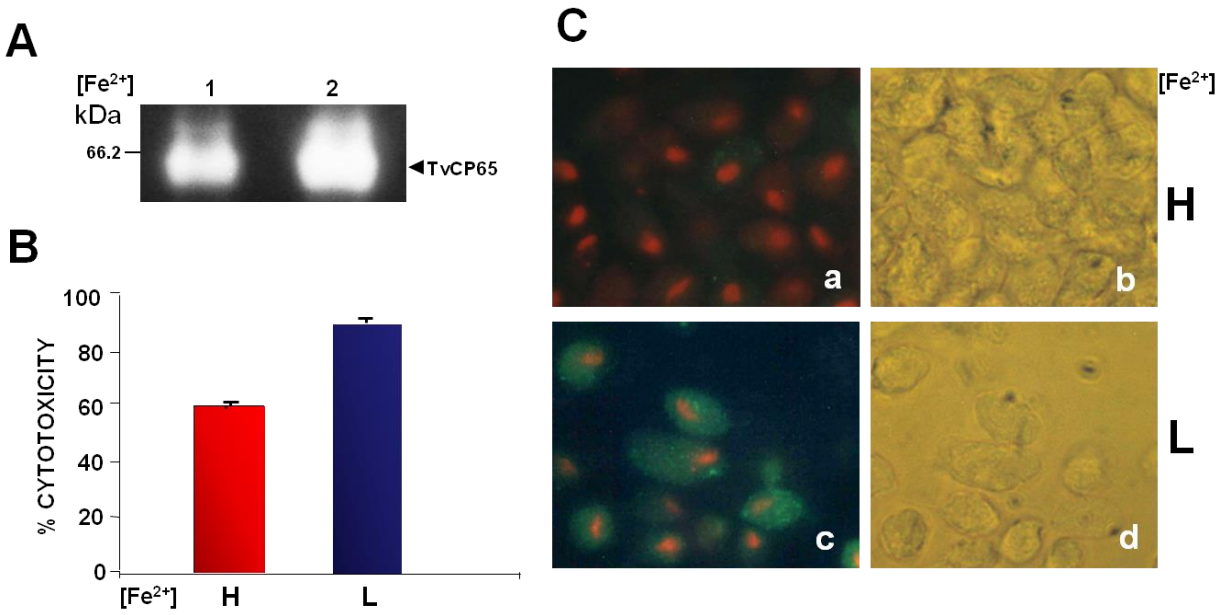

Figure 2. IRON EFFECT IN THE 65-kDa CYSTEINE PROTEINASE (TvCP65) THAT PARTICIPATES IN *T. vaginalis* CYTOTOXICITY TO TARGET CELLS.

A. TvCP65 (CP65) zymogram from high (H, lane 1) or low (L, lane 2) iron-grown parasites.

B. Cytotoxicity levels from the same parasites in A.

C. Indirect immunofluorescence with the anti-TvCP65 antibody (green) using the same parasites from section A; panels a and b, parasites grown in high iron (H); panels c and d, parasites grown in low iron (L); b and d, phase contrast from the same preparations. Nuclei (red) counter-stained with propidium iodide (a and c). 40× magnification [12,13**].**

The *flp-1* and *flp-2* genes that encode for proteins with homology to fibronectin [30] and the ***tvcp12* gene that encodes for a papain-like CP, TvCP12** [16,17,72,73,74,82], are also negatively regulated by iron **(**Figure 3**)**.

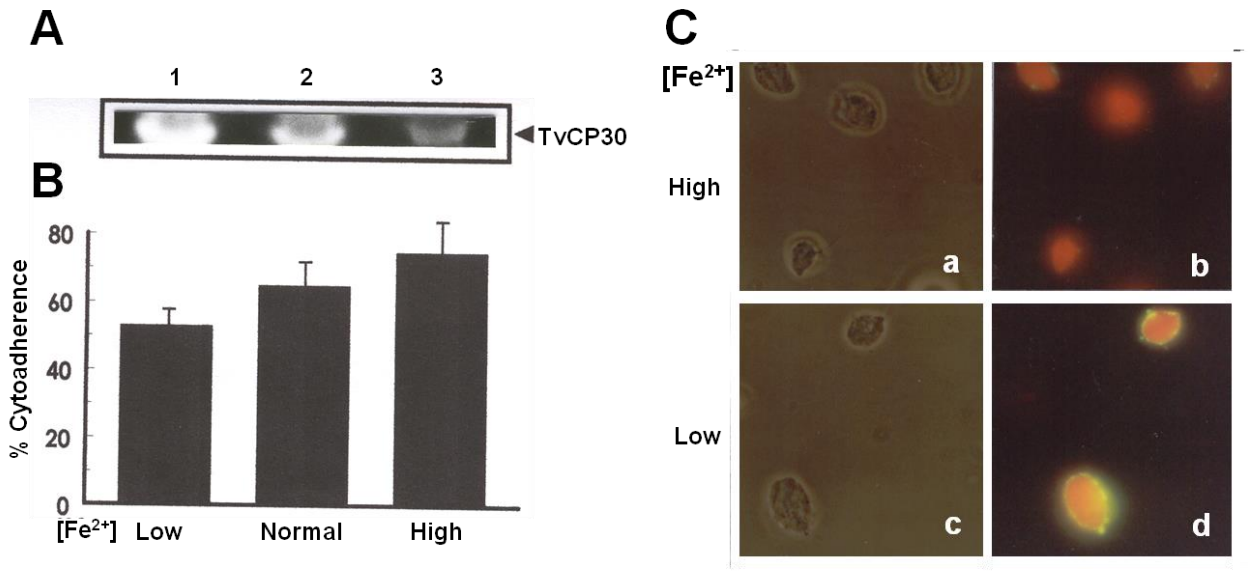

Figure 3. IRON EFFECT IN THE 30-kDa CYSTEINE PROTEINASE (TvCP30) THAT PARTICIPATES IN *T. vaginalis* CYTOADHERENCE.

A. TvCP30 zymogram from parasites grown in low (lane 1), normal (lane 2), and high iron conditions (lane 3).

B. Adherence levels from the same parasites in A.

C. Indirect immunofluorescence with the anti-TvCP30 antibody using the same parasites from section A: panels a and b, parasites grown in high iron; panels c and d, parasites grown in low iron; a and c, phase contrast of the same preparations. The nuclei (red) counter-stained with propidium iodide (b and d). 40× magnification [Fattel-Facenda, 1999, Master thesis].

The proteolytic activity of some secreted CPs of the 30-kDa region (CP2, CP3, CP4 and CPT) **increases in the absence of iron and triggers host epithelial cell apoptosis** [63]. Because in this study only the proteinase soluble fraction was used, it is impossible to know whether one or all four CPs identified by mass spectrometry are responsible for apoptosis induction. We cannot affirm either that all these CPs are down-regulated by iron, since there is another TvCP4 up-regulated by iron, which is localized in the parasite surface [105] and participates in hemolysis [Cárdenas-Guerra et al., 2012 our recent unpublished results].

Moreover, ***T. vaginalis*** **binds to vaginal mucin in low iron concentrations, then degrades it using some of its CPs; subsequently the parasite adheres to the cervical and vaginal epithelial cells** [69] (Table 2), which increases in iron-rich concentrations.

Table 2. PROTEINS AND GENES EXPRESSED IN THE ABSENCE OF IRON

Proteins or genes	Participate in:	References
flp-1 and *flp-2*	ND[a]	[30]
tvcp12, encodes for a cathepsin L-like CP	Cytotoxicity	[72,73,74]
TvCP39	Cytotoxicity	[48,49]
TvCP65	Cytotoxicity	[12,13]
TvCP30	Adherence and apoptosis	[16,17,63,82]
Tv44	IgA degradation	[89]
P270	Immunogen phosphorylation level	[10]
136 kDa LfR[b]	Lactoferrin capture	[66]

[a]ND, not determined; [b]LfR, Lactoferrin receptor.

It is interesting to mention that **iron differentially modulates the proteolytic activity of the 30 kDa CPs** (Figure 4A) [70] **and the transcript quantity** (Figure 4B). These CPs belong to at least two different clans: 1) the **papain-like** from clan CA and 2) the **legumain-like** from clan CD [70,71,72,118]. Further studies are required to elucidate the individual function of each CP (adherence, induction of apoptosis, cytotoxicity, complement resistance, mucin and tight junction degradation, etc.), and their particular mechanism of iron regulation.

These data suggest that **some of the negatively iron regulated molecules are expressed and participate during infection**, as occurs with the P270 protein negatively regulated by iron. P270 is an immunodominant antigen that undergoes phosphorylation and antigenic variation in *T. vaginalis* isolates infected with the double-stranded RNA virus (TVV). It is alternatively expressed on the parasite surface with the adhesins, and is part of the host immune-evasion response [10].

It is conceivable that this **differential parasite response to iron concentrations occurs *in vivo***, allowing the parasite to survive and adapt to the cyclic changes in the reproductive female

vagina, favoring a chronic infection. *T. vaginalis* requires great levels of adherence and resistance to complement during menstruation that increases the vaginal flow and exposes the parasite to complement.

T. vaginalis* also requires a greater destructing capacity during the menstrual cycle** when the iron concentration declines, trichomonads become more mobile, less adherent, and more cytotoxic to obtain iron from some epithelial cellular components such as **Cytochrome *c and **Ferritin** [3,20].

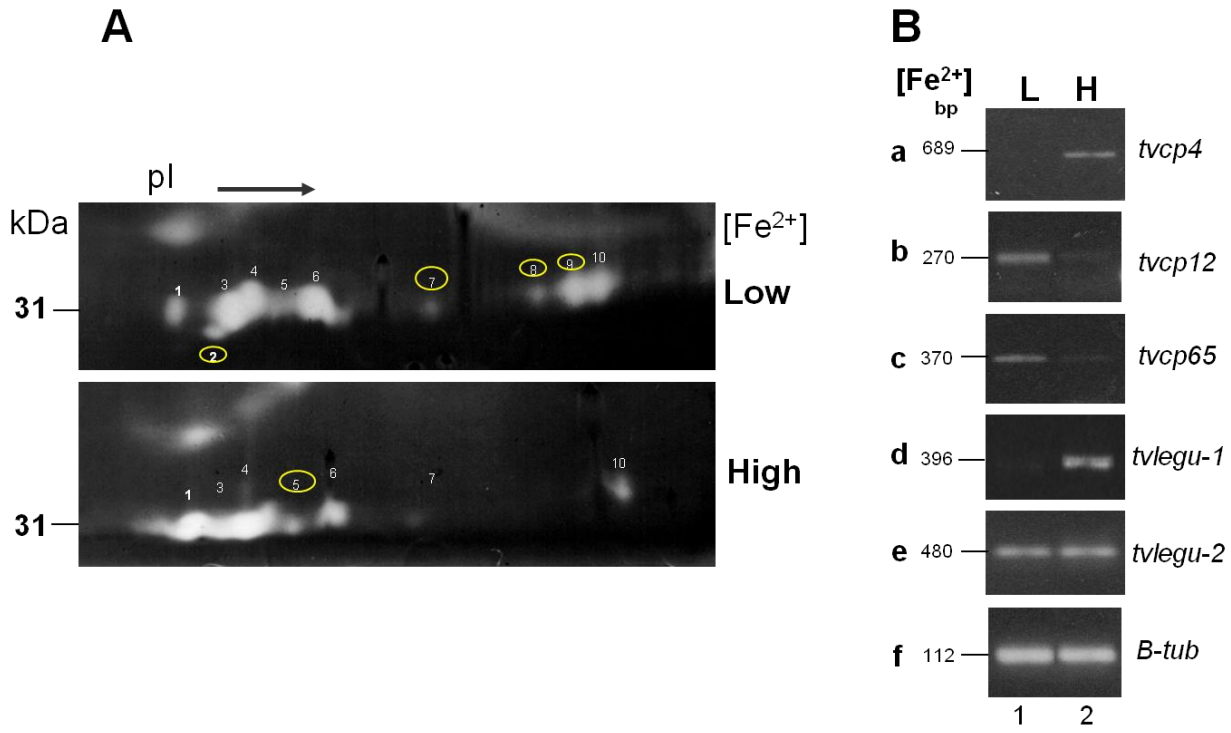

Figure 4. DIFFERENCIAL EXPRESSION OF *T. vaginalis* CYSTEINE PROTEINASES BY IRON.

A. 2-DE zymograms from iron-rich and iron-restricted parasite extract ([Fe^{2+}]).

B. RT-PCR assays of different proteinase genes using RNA from parasites grown under different iron concentrations (Low, L; High, H) and specific primers to amplify fragments of *tvcp4* (a), *tvcp12* (b), *tvcp65* (c), *tvlegu-1* (d), *tvlegu-2* (e), or *β-tubulin (β-tub)* (f), which was used as a quantity control (Adapted from Torres-Romero & Arroyo, 2009 [118].

2. *T. vaginalis* iron sources:

T. vaginalis uses multiple iron sources: free iron in ferrous form, Lf, Hemoglobin, Heme group, Cytochrome *c*, and Ferritin, but not Transferrin (Tf) [7,65,66] (Figure 5).

2.1. Lactoferrin

***T. vaginalis* specifically binds Lf through a surface receptor (LfR) and internalizes it with its receptor,** an increase in the intracellular enzymatic activity is observed, and **the receptor is recycled** [66,95]. Although the specific mechanism of Lf uptake is still unknown, the stoichiometric analysis shows that there are near **90,000 molecules of receptor per cell** [66,95]. **The LfR is a 136-kDa surface protein that is induced in iron-restricted conditions and is detected by patient sera, suggesting that is expressed during infection** [66] (Figure 5).

2.2. Hemoglobin

***T. vaginalis* also binds both Hemoglobin (Hb) and the Heme group through specific receptors, different from those used for Lf uptake** [7,65,67]. Hemoglobin and heme iron are alternative sources used by trichomonads for growth **and up-regulation of adhesin gene expression, increasing *T. vaginalis* adherence to immortalized vaginal epithelial cells** [3,7]. ***T. vaginalis* Hb and Heme group uptake is mediated by the AP65 and AP51 adhesins** [15] (Figure 5).

2.3. Other iron sources

This protozoan **uses several cells as iron sources: human erythrocytes, and cervical and vaginal epithelial cells**. *T. vaginalis* binds to the surface of these cells through its adhesins (12.5 and 25 kDa for erythrocytes and AP120, AP65, AP51, AP33, and AP23, for cervical and vaginal cells) and then lyses, or internalizes them. In the presence of erythrocytes or epithelial cells, trichomonads are able to grow and multiply in a medium lacking iron and lipids, strongly suggesting that ***in vivo* both human erythrocytes and epithelial cells are their natural iron and lipid sources** [65,66,67,68,84] (Figure 5).

Despite the multiple iron sources used by *T. vaginalis*, the mechanisms involved in iron release inside the parasite, the molecules responsible for and to store it are unknown, especially since this parasite lacks Ferritin. However, it is **suggested that iron could be stored in the multiple trichomonad iron-sulfur proteins** used for its metabolism, including **Ferredoxin**, as in *T. foetus* [111, 112].

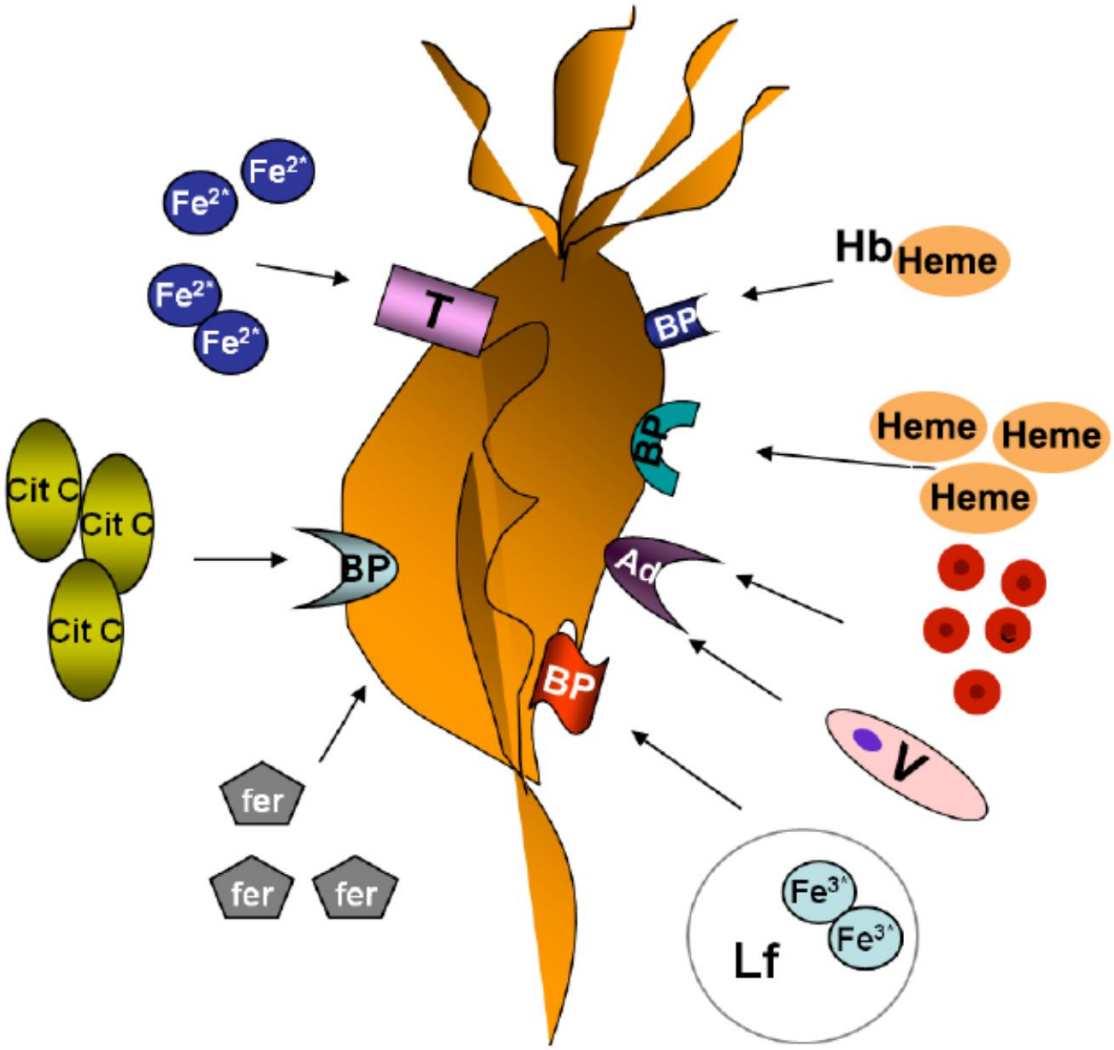

Figure 5. IRON SOURCES FOR *T. vaginalis.*

***T. vaginalis* uses multiple iron sources: free iron in ferrous form (F), Lactoferrin (Lf), Hemoglobin (Hb), Heme group (Heme), Cytochrome c (Cit c), and Ferritin (Fer), but not Transferrin. Besides, it can use several cells such as erythrocytes (red spheres) and epithelial cells (pink) as iron sources, but first needs to specifically binds to the surface of these cells using specific surface molecules named adhesins (Ad) or binding proteins (BP).**

3. Mechanisms of gene expression regulated by iron in *T. vaginalis.*

In support to the hypothesis that iron has a relevant role in the biology of this protozoan parasite, there are more molecules and properties of *T. vaginalis* reported to be positively or negatively regulated by iron.

However, little is known about the mechanisms that *T. vaginalis* uses for gene expression regulation by iron at both transcriptional and post-transcriptional levels.

3.1. Mechanism of transcriptional regulation by iron:

Currently, **a transcriptional iron-regulatory mechanism mediated by an iron-responsive promoter identified in the 5'-UTR of the *ap65-1* gene** [8,19,91] has been described. The *ap65-1* gene promoter activity in response to iron concentrations, growth conditions and multiple nuclear protein interaction was evaluated by **electrophoretic mobility shift assays (EMSA) and primer extension assays** using a DNA transfection system developed by Dr. Tai's group [91,92,93,119].

Multiple DNA motifs closely distributed in the iron-responsive region (-110/-54) **participate** in the positive transcriptional iron regulation of the *ap65-1* gene expression. Among them, **an iron-responsive promoter element** [(-98)AGATAACGA(-90)] **that overlaps to the 3' end with a Myb-like protein binding sequence (MRE)** [(-95)TAACGATAT(-87)] is included. Additionally, two overlapped MRE recognition elements (MRE-1/MRE-2r) and three T-rich sequences [(-110)ATTTTT(-105), (-78)ATTATT(-73), and (-59)ATTTTT(-54)] [91,92,93,118] are detected in the same region (Figure 6).

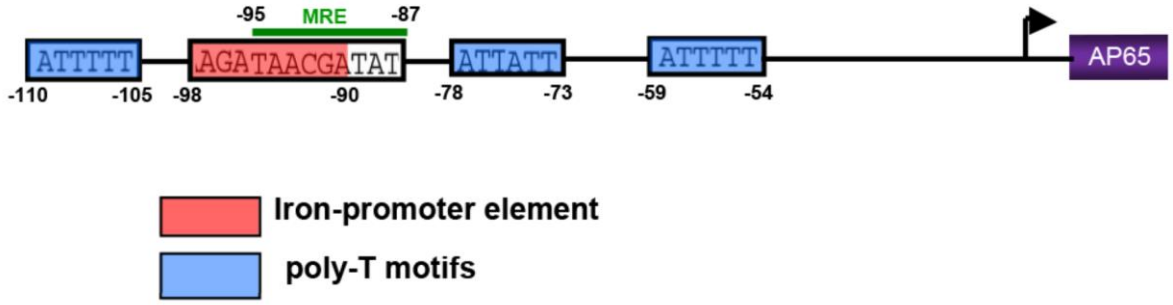

Figure 6. TRANSCRIPTIONAL IRON-REGULATORY MECHANISM IN *T. vaginalis.*

Multiple DNA motifs closely distributed in the iron-responsive region (-110/-54) participate in the positive transcriptional iron regulation of the *ap65-1* gene expression; among them, an iron-responsive promoter element [(-98)AGATAACGA(-90)] that overlaps to the 3'-end with a Myb-like protein-binding sequence (MRE) [(-95)TAACGATAT(-87)] is included. Additionally, two overlapped MRE recognition elements (MRE-1/MRE-2r) and three T-rich sequences [(-110)ATTTTT(-105), (-78) ATTATT (-73), and (-59)ATTTTT(-54)] are detected in the same region [91,92,93,118].

The transcriptional efficiency of the *ap65-1* gene changes with the variations in iron concentration and the parasite growth phase. Two recognition elements for **Myb-like** proteins, MRE-1/MRE-2r have antagonist functions in the iron-inducible regulation. Any of these elements is enough to repress the basal promoter activity, but together they activate the growth-related activity in cells that lack iron. **The flanking 5'- (AGTGAAGTGA) and 3'- (MRE-2f) sequences of the iron responsive region regulate**

basal transcription, allowing binding of nuclear Myb-like proteins. In the early logarithmic phase, the transcriptional activity is the lowest and in the middle logarithmic phase, is the highest one [91,92,93,118].

Myb1 is a 35 kDa nuclear protein regulated by iron that has R2R3 DNA-binding domains and binds to these promoter regions. This protein is a critical factor in the transcriptional regulation of the *ap65-1* gene through a differential selection of the multiple promoter sites, depending on environmental changes [92]. **Myb2** is another iron-responsive promoter-binding protein. It recognizes different sequences and preferentially binds to MRE-2f sequences than to MRE-2r. High iron concentrations repress *myb2* expression and the temporal activation/deactivation of the promoter that remains active in the absence of iron. **Myb2** is involved in the *ap65-1* gene transcription related with growth and iron; possibly by a differential promoter selection competed with other **Myb** proteins [93].

A question that remains to be solved is ¿How many of the genes that positively regulate trichomonads iron level for initiation of transcription, as *ap65-1* gene used as a model, have these regulatory IREs? Therefore, it is necessary to do an exhaustive and systematic bioinformatics analysis to identify other trichomonads genes positively regulated by iron that may have this iron-responsive promoter or variants of it.

Given that this promoter is absent in other currently identified genes that encode for iron up-regulated proteins, it is suggested that this parasite may have other iron-regulatory mechanisms, possibly at the post-transcriptional level. These mechanisms would be mediated by RNA-protein interactions that respond to the lack of iron by stabilizing the mRNA or blocking its translation, as in the IRE/IRP system responsible for the cellular iron homeostasis [94,116].

3.2. Mechanism of post-transcriptional regulation by iron:

In vertebrates, the cellular iron homeostasis is maintained through the coordinated regulation of capture, storage, and iron excretion. The three processes are mainly regulated at the **post-transcriptional level** through iron regulatory cytosolic proteins (**IRP or IRE-BP**) that bind to **iron responsive elements** (**IREs**) in the absence of iron. These elements are hairpin-loop structures in **the untranslated region** (**UTRs**) of **mRNAs** that coordinate the synthesis of several important proteins related to iron storage and uptake. This post-transcriptional mechanism is known as the **IRE/IRP system** [1,35,37,41,42,46,94,116].

IRPs are iron sensor proteins that bind to IREs, blocking or stabilizing the translation of some mRNAs in the absence of iron. For example, the Ferritin mRNA translation is blocked in the absence of iron due to the IRP-1 binding at the 5'-UTR IRE [94,116].

Alternatively, **these RNA-protein interactions at the 3'-UTR IREs of iron-down-regulated transcripts**, **as the Transferrin receptor (TfR), protect mRNAs of ribonuclease degradation**, increasing the transcript stability and allowing its translation. The result is seen as an increase in the translation of the

transferrin receptor in the absence of iron [46,50,58,94,116]. **IRPs also sense and respond to oxidative stress** [94,116] (Figure 7).

3.2.1. *T. vaginalis* has a post-transcriptional mechanism mediated by RNA-protein complexes similar to the IRE/IRP system.

Studies regarding molecular pathogenesis of *T. vaginalis* infection and iron regulatory mechanisms involved in gene expression have allowed our group to identify and characterize **a new adhesin (AP120)** (Figure 1A) [84] **and some proteinases** (**TvCP30, TvCP39, and TvCP65**) (Figures 2 and 3) [12,13,16,17,48,49,82] as virulence factors that participate in *T. vaginalis* adherence or cytotoxicity. These proteins are **differentially modulated by iron,** which is reflected in the virulence properties these factors participate.

Analysis of the iron effect on two CPs identified and characterized as virulence factors, TvCP30 and TvCP65, showed that **the proteolytic activity and surface localization of both CPs decrease in parasites grown in the presence of iron** (Figures 2A and 3A), as compared **with those grown in its absence** (Figures 2C and 3C) [13,82]. These results suggest that TvCP30 and TvCP65 are **negatively regulated by iron,** at least at the proteolytic activity and surface localization levels. Regarding the iron effect in the function in which these proteinases participate, it was found that **while iron negatively affects its cytotoxicity** (Figure 3B)**, positively affects its cytoadherence** (Figure 2A), possibly due to the iron up-regulation of the adhesins gene expression [68] that also participate in trichomonal adherence. We must not forget that most of the *T. vaginalis* virulence properties are multifactorial.

It should be point out that the proteolytic activity of the 30-kDa region is formed by multiple proteinases as previously mentioned and further discussed (Figure 4). **The 2-D zymograms** of the 30-kDa region in parasites grown in different iron concentrations show **a differential iron modulation** with a complexity not observed in the 1-D zymograms. In iron-depleted parasites, up to 10 spots with proteolytic activity are observed; while in iron-rich trichomonads only 6-7 spots are detected (Figure 4A). Besides showing that the 30-kDa band is formed by multiple proteinases belonging to two different families, **papains and legumains** [70,71,72,73,74,104], the results also show that these CPs differentially respond to iron during growth, a greater number of CPs in iron-depleted than in iron-rich parasites are observed (Figure 4A).

The effect of the differential iron modulation in the expression of these enzymes is also reflected in the amount of transcript. The RT-PCR assays of some papain-like and legumain-like genes show that the expression of *tvcp4* and *tvlegu-1* is up-regulated, *tvcp12* and *tvcp65* is down-regulated, and *tvlegu-2* apparently is not affected by iron (Figure 4B). **These data show that during growth iron can differentially modulate the expression of each gene, affecting virulence or other relevant functions accordingly in which the CPs participate during infection.**

The existence of a post-transcriptional regulation in *T. vaginalis* in which IRP-like cytoplasmic proteins (that recognize stem-loop sequences located in the iron-regulated mRNA transcripts) participate, might help

explain that iron at the same concentration differentially modulates the expression of some *T. vaginalis* proteins and virulence factors. Supporting this hypothesis, it has been demonstrated that in ***T. vaginalis* cytoplasmic extracts, RNA-binding proteins** with molecular weights of 135-, 110-, 60-, and 45-kDa **interact with the Ferritin consensus IRE** [23], and have affinity for stem-loop IRE-like structures of some mRNA of *T. vaginalis* [117,118] differentially regulated by iron, such as *tvcp4* [105] and *tvcp12* [72,73,74]. These trichomonad stem-loop structures bind to the human IRP [43,74,105] and *T. vaginalis* cytoplasmic proteins in the absence of iron. Data show that ***T. vaginalis* iron regulation is also accomplished by a post-transcriptional mechanism mediated by RNA-protein complexes, similar to the IRE/IRP system used by other organisms for iron homeostasis** (Figure 7) [94,116].

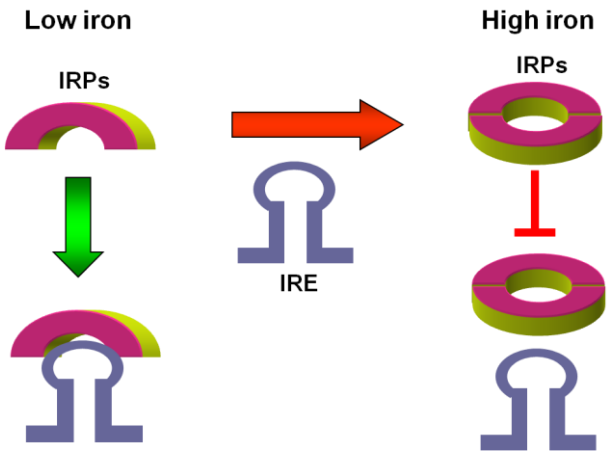

Figure 7. REPRESENTATIVE DIAGRAM OF THE ELEMENTS REQUIRED FOR THE IRON-MEDIATED POST-TRANSCRIPTIONAL REGULATION THROUGH THE IRE/IRP SYSTEM.

The RNA-binding proteins (IRPs) that recognize stem-loop structures (IREs, Iron Regulatory Elements) localized in the 5'- or 3'-UTR of some iron-regulated mRNAs.

IRPs are bifunctional cytoplasmic proteins: i) in the absence of iron, IRPs have RNA-binding activity; ii) in the presence of iron, IRPs lose their RNA-binding activity and show Aconitase activity or are degraded through the Proteosome pathway.

3.2.2. RNA-binding proteins in *T. vaginalis*.

By gel-shifting, UV cross-linking, and Northwestern blot assays using Ferritin IRE (IRE-*fer*), IRE-like *tvcp4*, or IRE-like *tvcp12* RNAs and *T. vaginalis* cytoplasmic extracts, two **ribonucleoprotein complexes were observed in the absence of iron**. At least three proteins of 98-, 60-, and 45-kDa, with affinity to human IRE-*fer* and IRE-like *tvcp4* structures (31 nt) were also found using HeLa cell extracts [23,105,117] (Figure 8).

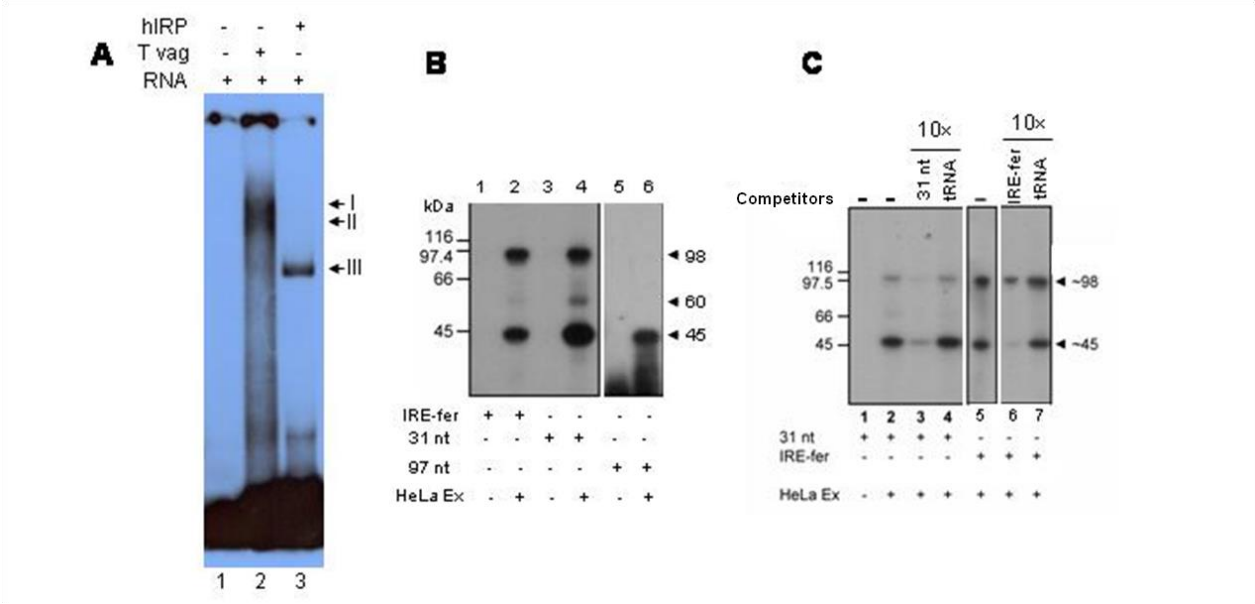

Figure 8. SPECIFIC RNA-PROTEIN COMPLEX FORMATION WITH THE *tvcp4* IRE BY GEL-SHIFTING (A), UV CROSS-LINKING (B), AND COMPETITION ASSAYS (C).

A. The gel-shifting assays were performed with the 94-nt *tvcp4* IRE transcript containing the ^{32}P-labeled IRE-like structures incubated with: lane 1, without protein (negative control); lane 2, *T. vaginalis* cytoplasmic extracts; lane 3, recombinant human IRP (rhIRP) protein; arrowheads show the ribonucleoproteic complexes (RNPC) I, II, III.

B. UV cross-linking assays performed with labeled transcripts; lanes 1 and 2, IRE-*fer*, lanes 3 and 4, 31-nt IRE-like *tvcp4*, or lanes 5 and 6, 97-nt mutant that lacks the first 11-nt of the IRE-like *tvcp4*- and does not make the typical stem-loop structure. Lanes 2, 4, and 6 were incubated with cytoplasmic extracts from HeLa cells as IRP source. Arrowheads show the protein bands of 98-, 60-, and 45-kDa associated to the radioactive IREs.

C. Competition assays for RNPCs formation between IRE-*fer* and the 31-nt IRE-like *tvcp4* and HeLa cell proteins. 10x molar excess of unlabeled RNA of specific homologous competitors (31-nt of the IRE-like *tvcp4* or IRE-*fer*) and tRNA as an unrelated competitor were used. The cross-linked complexes were analyzed in denaturing 10% SDS-polyacrylamide gels, Coomassie blue-stained, and visualized by autoradiography.

Noteworthy, the **molecular weights of the identified *T. vaginalis* RNA-binding proteins do not correspond to those of IRP-1/Aconitase or IRP-2 proteins.** This is not surprising since *T. vaginalis* lack mitochondria and the tricarboxylic acid cycle is absent, aconitase activity has not been detected, or the typical IRP proteins have not been found in the *T. vaginalis* genome. Thus, identification of IRE-binding proteins in *T. vaginalis* supports the existence of a post-transcriptional iron regulation mediated by a mechanism similar or parallel to the IRE/IRP system that has to be elucidated and these results may shed some light to the possible evolutionary relevance. Solving this will represent a relevant goal for our group.

3.2.3. IRE-like hairpin structures in *T. vaginalis*

The first 31-nt of the *tvcp4* transcript coding region form an IRE-like stem-loop structure (Figure 9C) similar to the Ferritin IRE (Figure 9A) and to the 75-kDa mitochondrial subunit of complex I (Figure 9B); however, the loop sequence (Figure 9C**, red letters**) is different comparing to the IRE consensus sequence (Figures 9A y B). Additionally, **in the 19-nt of the 3'-UTR of a negatively iron regulated gene (*tvcp12*) a stem-loop structure** (Figure 9D), **similar to IRE-*fer*** (Figure 9A), **was also found.** Both IRE-like transcripts bind to human IRPs and form two RNA-protein complexes (RNPC) similar to those observed with the IRE-*fer* probe (Figure 8). This was confirmed by UV cross-linking, competition, and the use of IRE-mutants assays. The formation of these complexes is specific because only the homologous RNA competed, but not the tRNA used as a negative control (**Data not shown**).

Analysis of mRNA secondary structures [125] of the 3'-UTR of other negatively-iron regulated genes, *p270* [10] and *flp-1* [30] predicts formation of IRE-like stem-loop structures (Figures 9E and F).

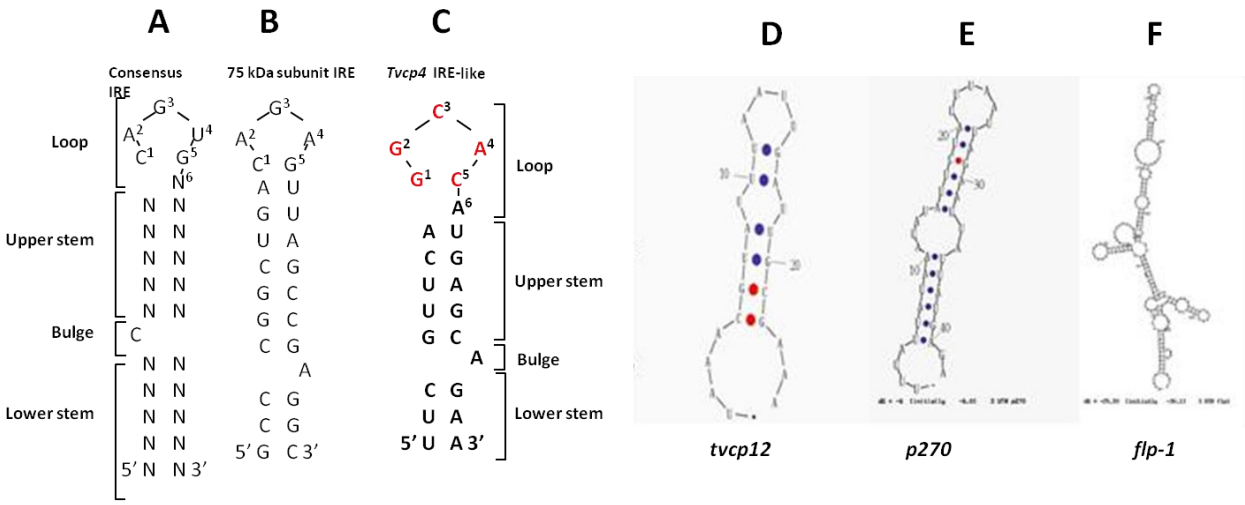

Figure 9. PREDICTION OF STEM-LOOP IRE-LIKE STRUCTURES OF THE *tvcp4, tvcp12, p270* AND *flp-1* 5'- OR 3'-UTR mRNAs.

A. Secondary structure of the human Ferritin IRE (IRE-*fer*) confirmed by NRM.

B-F. Prediction of IRE-like stem-loop structures by the Zuker program [125] of the 75-kDa mitochondrial subunit of the respiratory chain complex I and *T. vaginalis tvcp4, tvcp12, p270*, and *flp-1* (F) 5'- or 3'-UTR mRNA, respectively.

3.2.4. Hypothetical model of the post-transcriptional iron regulation in *T. vaginalis*.

Based on our results we propose a hypothetical model of the post-transcriptional iron regulation mechanism in *T. vaginalis* mediated by RNA-protein interactions similar to the IRE/IRP-like system [94,116] for genes that are up- or down-regulated by iron such as *tvcp4* and *tvcp12* (Figure 10 and Figure 11, respectively). The up-

stream region of the *tvcp4* gene lacks the iron-responsive promoter sequence involved in the positive iron regulation at the transcription initiation level, previously described for the *ap65-1* gene encoding the AP65 adhesin positively regulated by iron [91,92,93,119].

In the absence of iron, the cytoplasmic IRE-like-binding proteins will interact with stem-loop structures present in the 5'- or 3'-UTR of some CP mRNAs, such as *tvcp4* and *tvcp12*, respectively (Figure 10), blocking the translation of the *tvcp4* mRNA and stabilizing the *tvcp12* mRNA, allowing its translation.

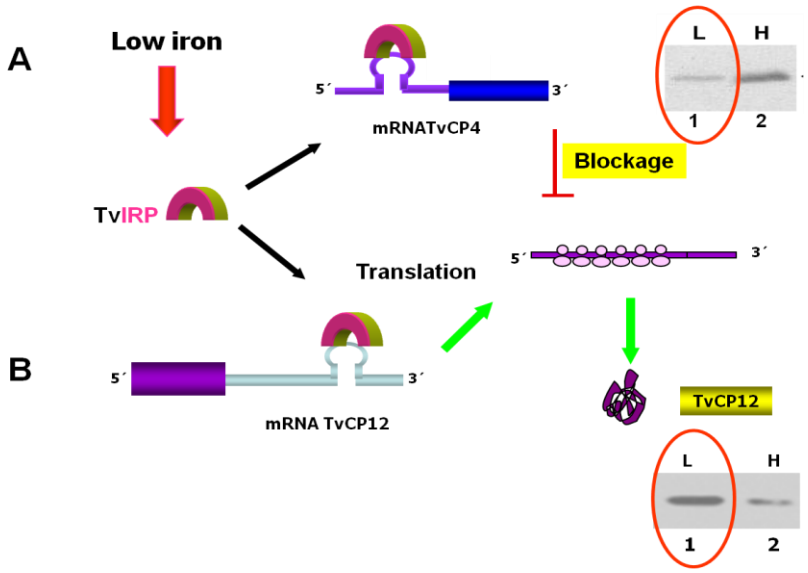

Figure 10. HYPOTHETICAL MODEL TO EXPLAIN THE MECHANISM OF NEGATIVE IRON REGULATION, OCCURRING AT THE POST-TRANSCRIPTIONAL LEVEL IN *T. vaginalis.*

In the absence of iron, the cytoplasmic RNA-binding proteins with the correct conformation will bind to stem-loop structures (IRE-like) located in the 5'- or 3'-mRNA of target genes, generating two different effects:

A. mRNA translation will be inhibited by the RNA-protein complex, by blocking binding of ribosomes; thus the TvCP4 protein will be reduced or undetected in trichomonads.

B. Translation of mRNA possessing the IRE-like structure at its 3'-end, like *tvcp12*, will be activated by the RNA-protein complex formation that will stabilize the mRNA, allowing its translation. Thus, the gene product (TvCP12) will be observed in the parasite.

Genes positively regulated by iron, such as *tvcp4*, must have an IRE-like structure in the mRNA 5' end, to which RNA-binding proteins will interact in the absence of iron, blocking the ribosome anchoring and hence the protein synthesis (Figure 10). Instead, in the presence of iron no RNA-protein interactions will occur at the 5'-UTR. Thus, the mRNA will be translated and the resultant proteins will be observed (Figure 11). The IRE-like *tvcp4* mRNA structure was found in the coding region (first 31 nt), it binds *T. vaginalis* RNA-binding proteins in the absence of iron, and less amount of TvCP4 was observed possibly due to protein

synthesis blockage (Figure 10A) [105], while in the presence of iron there is no RNA-protein complex formation and greater amount of TvCP4 will be observed (Figure 11A) than in iron-depleted conditions.

In **the negative iron regulated genes such as *tvcp12***, the stem-loop structures should be found in the 3'-UTR mRNA that will interact with IRE-like-binding proteins in the absence of iron, conferring stability to the transcripts, and allowing the protein synthesis to occur (Figure 10). However, this RNA-protein interaction will not occur in the presence of iron; thus the mRNAs will be degraded and no protein synthesis will occur (Figure 11).

The IRE-like structure of *tvcp12* at the 3'-UTR of its mRNA interacts with RNA-binding proteins in the absence of iron, stabilizing its transcript for TvCP12 translation (Figure 10B). In iron-rich concentrations, no RNA-protein interactions occur, the transcript degrades, and the amount of TvCP12 decreases (Figure 11B) [72,73,74]. This model can explain the differential iron regulation observed for multiple genes at the same iron concentration, affecting *T. vaginalis* virulence.

The presence of i) IRE-like sequences in some mRNA differentially regulated by iron, ii) cytoplasmic RNA-binding proteins, and iii) their functionality support **the existence of a post-transcriptional iron regulatory mechanism in** *T. vaginalis*. This regulatory mechanism **is mediated by RNA-protein complex formation, similar to the IRE/IRP system required for cellular iron homeostasis.** Interestingly, the IRE-like structures and cytoplasmic RNA-binding proteins identified in *T. vaginalis* differ from the consensus elements of this system: i) IREs do not have the canonic loop sequence [94,116] (Figure 9) and ii) the size of the RNA-binding proteins do not correspond to the typical IRP-1 and IRP-2 proteins (Figure 8A), which makes more attractive to study this iron regulatory system in *T. vaginalis*.

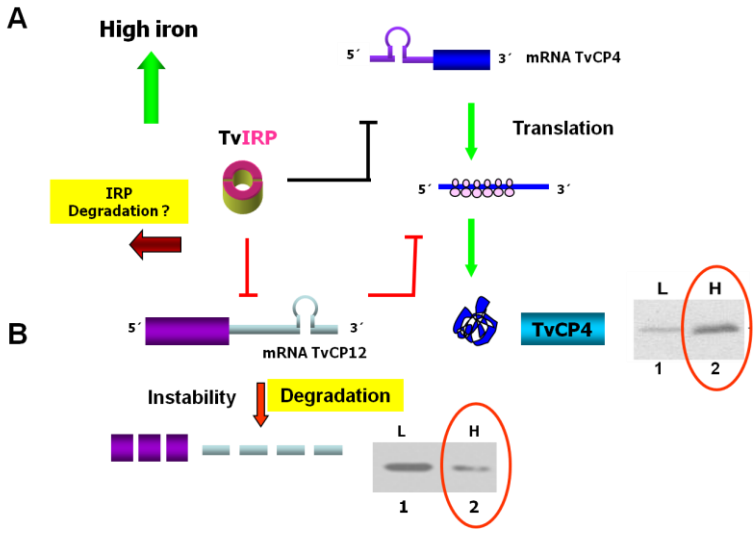

Figure 11. HYPOTHETICAL MODEL OF THE POSITIVE IRON REGULATORY MECHANISM AT THE POST-TRANSCRIPTIONAL LEVEL IN *T. vaginalis*.

In the presence of iron, the RNA-binding proteins will not interact with the stem-loop structures (IRE-like) in the mRNA ends of the target genes, due to protein conformational changes or degradation that will have two effects:

A. If the IRE-like structure is at the 5'-end, as *tvcp4*, the mRNA will be translated and the corresponding protein (TvCP4) will be detected in the parasite.

B. If the IRE-like sequence is at the 3'-UTR, as *tvcp12*, the mRNA would be degraded by the parasite RNases and less or no product (TvCP12) would be observed in the parasite.

This type of post-transcriptional iron regulatory mechanism in *T. vaginalis* can help to explain the paradox observed in the iron response for different molecules and functions at the same iron concentration to which the parasite is exposed during infection. Variations in the iron concentrations somehow reflect the situation faced by *T. vaginalis* in the vagina during infection, being exposed to hormonal and nutrient changes (iron concentrations) throughout the menstrual cycle and shows the adaptative capacity of this protozoan parasite to survive and cause a chronic infection.

Iron levels allow the parasite to maintain a **chronic infection** by modulating the differential expression of its genes and its virulence properties during the menstrual cycle, **showing variations in the levels of adherence and cytotoxicity, induction of apoptosis, immune-evasion response, etc**., which depend on iron concentrations. This phenomenon is also observed in other microorganisms of the urogenital tract as *Candida albicans* in which has recently been described a differential expression of several iron-dependent genes during growth [64].

The relevance of this finding lies in the help it will bring in understanding the effect that iron menstrual cycle variations causes on *T. vaginalis* virulence regulation and its possible application to improve diagnosis and treatment of human trichomonosis.

Based on these results, **it is possible that we are dealing with the origin of a parallel iron regulatory system** that regulates virulence factors of some anaerobic parasites as primitives as *T. vaginalis*, which lacks mitochondria and inhabits an environment with cyclic iron fluctuations, such as the vaginal microenvironment. During species evolution, another post-transcriptional iron regulatory mechanism was adjusted and used for adaptation to changing iron concentrations in the host microenvironment, giving place to the specialization of the highly conserved IRE/IRP system to control the intracellular iron homeostasis.

This IRE/IRP post-transcriptional mechanism will allow the coordinated regulation of proteins involved in iron metabolism homeostasis as preserved among the species that have Kreb's cycle and aconitases or aconitase-like proteins [58,101], from bacteria [11,114], protozoa [76,77,80,103], insects [59,85,123,124], to higher eukaryotes [14,44,45,53,56,97,122], which would explain the selection and conservation of consensus loop sequences of canonic IREs in this type of genes as well as the key role played by highly conserved regulatory proteins, IRP-1 and IRP-2.

This **parallel post-transcriptional mechanism mediated by RNA-binding protein complex formation** described in *T. vaginalis* could be responsible for iron regulation in other virulence genes as adhesins, CPs, and others not yet identified that participate in the virulence of a protozoan as primitive as *T. vaginalis* and possible in other amitochondriate pathogens such as *T. foetus, Entamoeba histolytica,* and *Giardia lamblia*.

4. Iron in *Tritrichomonas foetus*.

Tritrichomonas foetus is the protozoan parasite of veterinary importance responsible for **bovine trichomonosis**, a sexually transmitted infection that causes infertility and abortion in cattle [36]. This parasite is related to the *T. vaginalis* human pathogen. Therefore, studies on bovine trichomonosis might help understand the mechanisms of *T. vaginalis* pathogenicity and future female trichomonosis protection strategies. **Virulence factors similar to those of *T. vaginalis* have been detected** by *in vitro* studies**, including CPs and adhesins that interact with host cell proteins and factors involved in adherence to bovine vaginal epithelial cells.**

Although the animal model of *T. vaginalis* infection of estrogenized mice is useful to study virulence factors [27], **the bovine model allows immunity studies in the natural host.** Additionally, the bovine model has proven to be an **effective system for vaccination testing for both prophylaxis and treatment of the *T. foetus* infection.** The specificity and isotype of the protective antibodies developed in this model have been defined. This could facilitate vaccine development strategies against human trichomonosis [27].

4.1. Iron in *T. foetus* virulence.

***T. foetus* has also high iron requirements** (50-100 μM) as *T. vaginalis,* modulating its growth rate in *in vitro* culture. **When *T. foetus* is cultured in medium without iron, an increase in the levels of cytoadherence and cytotoxicity, as well as in the activity of some CPs is shown.** Therefore, it is suggested that iron is also an extracellular signal for *T. foetus* that seems to modulate the host-parasite interaction, but contrary to what happens with *T. vaginalis* [21,81].

***T. foetus* uses extracellular CPs to quickly degrade host proteins (fibrinogen, fibronectin, albumin, Lf, IgG1, and IgG2), and with less efficiency IgM, IgA, and Tf.** This proteolytic activity is inhibited by E-64 and alpha-2 macroglobulin. Due to the nature of the degraded proteins it is suggested that the CPs could participate in the host-parasite interaction and therefore be part of the *T. foetus* virulence mechanisms for nutrient acquisition and degradation of some host-defense proteins [113].

In the ICR mouse model intraperitoneally inoculated with the KV-1 strain of *T. foetus,* **iron increases virulence causing from 5% to 80% mortality** after ferric ammonium citrate administration (100 mg/Kg per day) for at least three days after parasites inoculation. This increase in virulence was due to a rise in the

peritoneal parasite multiplication, which was not seen in untreated mice used as control. *In vitro* studies showed that the KV-1 strain has a low efficiency in iron accumulation through Tf and low molecular weight compounds [Fe(III)-Nitriloacetic acid]. These data suggest that the **parasite iron acquisition mechanisms should be considered part of the virulence properties necessary for the infection development by *T. foetus*** [62,108,109].

4.2. Iron acquisition mechanisms in *T. foetus*.

***T. foetus* also uses multiple iron sources, Lf, Tf, Hemin, Fe(III)-Nitriloacetate chelating compounds, and some xenosiderophores** (Figure 12). The high iron requirements of *T. foetus* are mainly satisfied from bovine Lf, followed by human and bovine Tf (regarding to its binding capacity).

***T. foetus* binds Lf through receptor-mediated (LfR) hydrophobic and charge-related interactions** [40]. This protozoan has nearly 1.7×10^5 binding sites for Lactoferrin with a **Kd of 3.6 μM; this interaction is time-dependent and is saturable at 4 °C. At 37 °C, *T. foetus* binds a greater amount of Lf, which is then internalized.** Despite the binding of *T. foetus* to Tf is not specific nor shows saturation kinetic, both proteins are iron sources that stimulate *T. foetus* growth. The capacity of this parasite to remove accumulated iron in both proteins is similar (495 and 577 pmoles Fe/mg of protein, respectively) and requires an acid pH to achieve it (pH 5.6).

The iron acquisition mechanism for these two host proteins is different: 1) through the specific binding and internalization of LfR and cytoplasm release and 2) from Tf, iron acquisition depends on extracellular release and ligand reduction [111]. Even though several *T. foetus* surface proteins of 22-, 49-, 55-, 72-, and 155-kDa have been detected to possess affinity to Lf, it is suggested that the LfR could be the 155-kDa protein, while the other proteins could correspond to processing products that share the Lf-binding site. However, it cannot be discarded the existence of multiple Lf-binding proteins or multimers of the same protein [109] (Figure 12).

***T. foetus* also acquires iron from Hemin, probably through the Heme Oxygenase** because biliverdin remnants are found in the medium when Hemin is used as the only iron source. However, it seems that neither the iron uptake nor the Ferrireductase activity are regulated by the presence of iron and copper in the culture medium [107,109] (Figure 12).

Although *T. foetus* can also acquire iron through Fe(III)-Nitriloacetate chelating compounds (Fe-NTA) in a saturable way (Km = 2.7 μM, with a Vmax = 21.7 fmol. μg^{-1}. min^{-1}) on a time- and temperature-dependent mode, reduction of extracellular ferric to ferrous iron is required. This mechanism is very similar to the one needed to release iron from Tf. The data show that ***T. foetus* has at least two independent mechanisms for iron acquisition** [112].

***T. foetus* also acquires iron from siderophores** such as: **Ferrioxamine B, Ferrichrome, Triacetylfusarinine, Coprogen, Enterobactin, and Pyoverdine, through a non-saturable transport.** This parasite can grow in the presence of these siderophores as the only iron source in the culture medium. **Iron from siderophores is captured by Ferredoxin.** This uptake does not involve an extracellular reductive dissociation of ferric chelators; however, *T. foetus* has some Ferrireductase activity over ferric citrate [106,107,108,109].

It has been proposed that the uptake of multiple siderophores chemically unrelated is efficient but unspecific, possibly by **pinocytosis, and the intracellular iron is released in acid intracellular vesicles. This was confirmed by using siderophore fluorescent analogs. Their accumulation was detected in small intracellular vesicles with a highly acidic environment** [106,107,108,109] (Figure 12). Additionally, **these studies revealed Ferredoxin as the most important iron-binding molecule in the hydrogenosome; possibly acting as an iron storage molecule, equivalent to Ferritin in other organisms [106].**

5. Iron-sulfur proteins in *T. vaginalis* and *T. foetus.*

T. vaginalis and *T. foetus* have high exogenous iron requirements for an adequate growth and metabolism [39], especially for hydrogenosome function. **The main function of hydrogenosomes is the transformation of malate or pyruvate to H_2, CO_2, and acetate by an ATP synthesis associated to a metabolic pathway. This pathway depends on the activity of iron-sulfur (Fe-S) proteins such as PFOR, Hydrogenase, and Ferredoxin** [86].

These enzymatic activities, as well as H_2 and acetate production decrease or disappear in the absence of iron, including the hydrogenosomal Malic enzyme activity, even though it is not a Fe-S protein. This deficiency in the activity of hydrogenosomes is compensated by the cytosolic pathway activation, mediated by pyruvate to ethanol conversion through the acetaldehyde pathway. This metabolic switch is completely reversible once the iron concentrations in the medium are re-established after 3 h.

The reduction in the hydrogenosomal activity is due to a decrease in the PFOR, Malic enzyme, and Ferredoxin protein levels associated to a reduction of their mRNA synthesis, which demonstrates that iron controls the expression of these hydrogenosomal enzyme genes at the transcriptional level in *T. foetus* [120] and in *T. vaginalis* [51, 52]. It is possibly due to a high dependence and relevance of the Fe-S proteins such as Ferredoxin, PFOR, and Hydrogenase involved in pyruvate metabolism for energy production.

The main part of this metabolic pathway occurs in the hydrogenosome, a double membrane organelle that lacks DNA, but replaces mitochondria for ATP production [86].

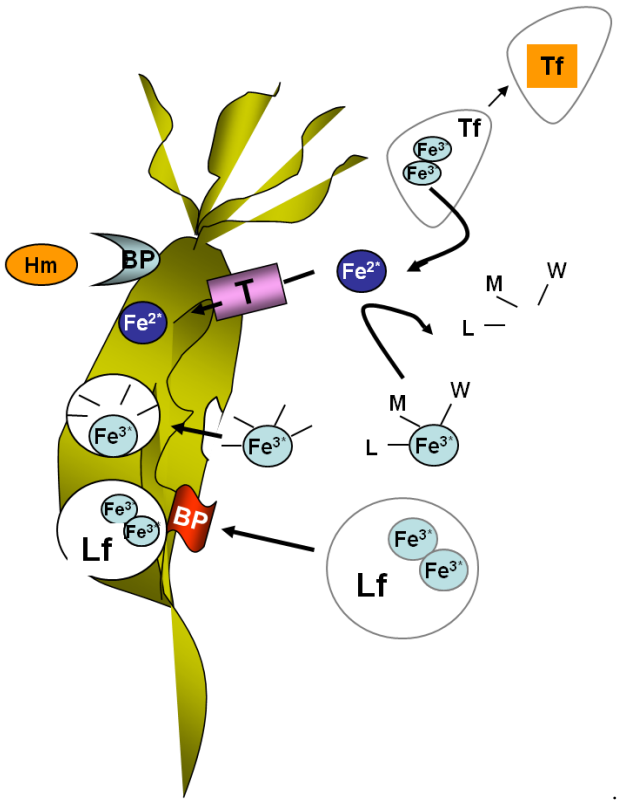

Figure 12. IRON SOURCES FOR *T. foetus*.

***T. foetus* also uses multiple iron (Fe) sources: Lactoferrin (Lf), Transferrin (Tf), Hemin (Hm), Fe(iii)-Nitriloacetate chelator compounds (Fe-NTA), and some siderophores.**

Iron availability has a sharp effect on carbohydrate metabolism of these two amitochondriate protozoa**. In the absence of iron, hydrogenosome metabolism dramatically decreases and the major glucose degradation product is ethanol instead of molecular hydrogen, due to Pyruvate Decarboxylase overexpression, the limiting enzyme in ethanol production** [86,120]. In the presence of iron, an increase in the transcripts and Fe-S protein concentrations [including Ferredoxin, PFOR, Hydrogenase, and Malic enzyme (in spite of lacking Fe-S cluster] is observed, which is related with an *in vivo* rise of hydrogenosomal metabolism and hydrogenosome membrane potential [22,28,39,51,52,55,57,79,83,120,121].

***T. vaginalis* and *T. foetus* are part of the amitochondriated protozoa that together with *G. lamblia* and *E. histolytica* represent the earliest organisms in the eukaryote evolution.** This explains the marked differences in the energy metabolism in comparison with mitochondriated cells. These differences are reflected in: i) the metabolic functions of inorganic pyrophosphate, ii) the main role of Fe-S proteins in certain metabolic steps and iii) in the electron elimination generated in the metabolism of hydrogenosomal organisms in the form of molecular hydrogen; iv) the lack of the cytochrome-mediated electron transport and v) the

phosphorylation linked to the electron transport. This is why some reactions are catabolized by proteins for which there are not homologous for similar reactions in eukaryotes [86].

Finally, things worth knowing are that the **trichomonadids also possess the necessary enzymes for biosynthesis and assembly of the Fe-S group as**: Frataxin [34,75], a homolog of the bacterial and mitochondriated organisms Cysteine disulfurase, TviscS-1 and TviscS-2 [110], and an Isd11 with a similar function to a proteobacterial Cysteine disulfurase [100].

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Chapter 17

Entamoeba histolytica requires iron from the host to produce amoebiasis

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Abstract

In this chapter, the importance of iron for *Entamoeba histolytica* trophozoites (amoebae) is analyzed. This parasitic extracellular protozoan causes amoebiasis, a human disease of the large intestine. Amoebae can spread to other organs, mainly the liver causing hepatic abscesses. Amoebiasis leads to high morbidity and mortality in developing countries.

We discuss the results of other research groups and ours about the relationship of iron and amoebic virulence. Thereafter, we show the binding to and endocytosis of four distinct human iron-proteins by *E. histolytica*: holoLactoferrin (holoLf), holoTransferrin (holoTf), Hemoglobin (Hb), and Ferritin (Ft). This parasite uses all of these proteins as iron sources for its growth when cultured in axenic media (without bacteria or associated protozoa); in these media the iron-containing protein is added instead of the ferric citrate.

To use these iron sources in vitro, and most like this occurs in vivo, *E. histolytica* counts with at least one receptor that recognizes and endocytoses each protein. Regarding holoTf, one receptor is the Acetaldehyde/ Alcohol dehydrogenase-2, an enzyme that requires iron for its activity. Once endocytosed, the iron-containing proteins travel through the endosomal/lysosomal route in order to be degraded and utilized as nutrients.

In mammals, Lf can exist in two different iron-saturated states, mainly depending on the diet: holoLf, with one or two atoms of ferric iron, and apoLf, free of iron. Amoebae are able to recognize and differentiate these two molecules of Lf *in vitro*, and in a similar way it might occur during their stay in the body. Interestingly, we found that Lf causes two different effects in *E. histolytica* depending on its iron saturation: when holoLf is in contact with amoebae, this ferric protein promotes the growth of parasites; but in the form of apoLf, it causes the death of amoebae. In addition, apoLf was found able to cure hepatic abscesses when it was used for disease treatment in a hamster model, and to cure intestinal amoebiasis in a mouse model.

In the human large intestine, amoebae usually engulf bacteria and exfoliated cells or their debris. Several host cellular and bacterial proteins such as enzymes, cytochromes and Ft contain iron that can be captured and utilized by amoebae; Ft is a protein capable of sequestering thousands of iron atoms. Since apoLf is normally found in the intestinal mucosa, this protein can be amoebicidal, but in form of holoLf it could contribute with iron to the multiplication of parasites in that site.

In further stages of amoebiasis, when the amoebae have destroyed the intestinal mucosa and invade the colon, they phagocytose erythrocytes and in this time intracellular Hb could serve as an excellent iron source for the parasites. Amoebae can also invade other organs such as the liver and lungs; when this occurs, trophozoites could utilize serum holoTf and Hb, as well as Ft from hepatocytes.

Key words: amoebiasis, amoebicidal, caveolin-like, clathrin, endocytosis, endosomes, *Entamoeba histolytica*, ferritin, hemoglobin, iron, iron source, lactoferrin, lysosomes, transferrin.

1. Amoebiasis

Human amoebiasis is a parasitic infection caused by *Entamoeba histolytica,* an extracellular protozoan. Cysts of this parasite are transmitted through the fecal-oral route by contaminated water or food [1]. Intestinal a**moebiasis is mainly characterized by lesions in the colon.** The symptoms are fever, abdominal pain, dysentery, and ulcerative colitis with mucous and blood. Amoebae can spread to other organs like the **liver**, **lungs**, **kidneys,** and **brain** because of its proximity or by hematogenous route. **Hepatic amoebiasis is characterized by liver abscesses that can be fatal** [2, 3, 4].

Amoebiasis is the third leading cause of death due to parasites, after **malaria and schistosomiasis**. This disease presents a high index of morbidity and mortality, mainly in developing countries [4]. According to the **World Health Organization (WHO)**, 500 million people are infected with amoebae; a 10% of them have virulent *E. histolytica* leading to 40,000-100,000 deaths annually. In Mexico, there are approximately 16 million people infected with *Entamoeba*; 1.3 million people have *E. histolytica* and 1000-3000 people die per year, mainly due to intestinal ulcers and **liver abscesses** [2, 4, 5].

E. histolytica* infects only human beings**. There is also non-pathogenic species of *Entamoeba* such as ***E. dispar* and *E. moshkovskii, which are morphologically indistinguishable from *E. histolytica* [5]. The life cycle of *E. histolytica* consists of two main stages: the **trophozoite (also called amoeba) or invasive stage, and the cyst or infective stage.** The cysts can tolerate the acidic pH of the stomach and then they excyst in the terminal ileum. **Trophozoites colonize the large intestine and in later phases of amoebiasis they invade the epithelium and mucosa** [1, 2, 3].

Adherence to colonic mucosa leads to a continued reproduction of parasites and to tissue damage by the products secreted by the amoebae. In response to unknown stimuli, the amoebae undergo morphological and biochemical changes leading to the formation of new cysts, which are eliminated in feces, closing the cycle [1, 3, 4].

One of our fields of research is the study of the binding to and endocytosis of iron-containing proteins by *E. histolytica* trophozoites; amoebae may well use these proteins for their growth and invasion inside the host. As this amoeba can invade several human organs, and **in each of these organs exist different proteins with iron, thus these proteins are potential iron sources for *E. histolytica*** [6, 7, 8, 9].

2. Iron (ferrous and ferric) and *E. histolytica*

Like most living organisms, *E. histolytica* needs iron for its metabolic functions. This parasite requires large amounts of iron for living, because in amoeba metabolism participate iron-sulfur proteins for electron transference that catalyze the oxidation of **dinucleotides** with reduced adenine **(NADPH and NADH)**. Some

iron-dependent crucial enzymes include the **Acetaldehyde/Alcohol dehydrogenase-2, Diaphorase and Ferredoxin** [10, 11, 12].

Iron is also present in the amoebic **Superoxide Dismutase (SOD),** an important enzyme that avoids the toxicity of the superoxide radicals produced by macrophages [13]. Furthermore, **iron plays an important role in the amoeba resistance to metronidazole,** because when the iron concentration increases within the amoeba cell, there is an increment of SOD expression and in this way the amoeba becomes resistant to metronidazole [14].

The culture medium generally used for the amoeba axenic growth is BI-S-33. It contains **ferric ammonium citrate** in a concentration of 90-100 μM of Fe [15]. In this medium, the serum **Transferrin** (**Tf)** also contributes with its iron content, as well as the Fe traces from chemical reagents. The amount of iron required for the *in vitro* growth of *E. histolytica* trophozoites has been studied since 1965, and apparently depends on the amoebic strain [16]. *E. histolytica* can utilize iron in the two states, **ferrous (Fe^{+2})** and **ferric (Fe^{+3})** [16, 17].

The use of both iron-oxidation states indicates that *E. histolytica* has several options for scavenging iron from the environment. Moreover, the iron-chelating compound 7-iodo-8-hydroxyquinoline -5-sulfonate inhibits the growth of amoeba, suggesting that this metal is of vital importance for the parasite [16]. When iron is removed from the medium by using the resin **Chelex-100** (the residual iron concentration is <20 μM Fe), this parasite is not able to grow and the cultures die after the second transfer, when the cellular iron reserves are exhausted [6, 8, 9, 18]. Therefore, *E. histolytica* has an absolute necessity of iron as well as other protozoa have.

Disease of human amoebic liver abscess (ALA) may be reproduced in the **liver of Syrian golden-hamster**; therefore the virulence of *E. histolytica* is maintained in this model. In 1978 Dr. Diamond showed that **hamsters fed ferrous gluconate had high incidence and severity of liver lesions,** and concluded that iron is an important nutrient for this amoeba. In addition, patients suffering from ALA showed a hypo-ferrhemia state in serum, similar to that presented in bacterial infections, concluding that **the nutritional immunity by iron is also present in amoebiasis** [19].

The iron regulation by *E. histolytica* has been studied in two forms:

1. By **expression of different protein patterns either in medium with iron deficiency or overload.** This phenomenon indicates that *E. histolytica* possesses an iron stress response [17].

2. By **differential gene expression under iron deficiency**. Using the **mRNA differential display technique**, researchers found some genes involved in translation, such as that encoding for the **elongation factor EF-1*α***, and five ribosomal proteins, suggesting that **a low iron level increases the demand for translation machinery.** Also, a gene encoding for a **cysteine-proteinase** was found [20]. **Proteinases have**

been exhaustively studied and are considered as virulence factors, allowing the parasite to destroy and invade host regions such as epithelial tissues and mucous membranes of the colon [20, 21]. In the liver these types of enzymes help the parasites to survive [22, 23, 24, 25].

3. Host Proteins used as iron sources and their endocytosis by *E. histolytica* trophozoites

Now, we will analyze the use of four host proteins that contain iron, by *E. histolytica* trophozoites:

1) **Lactoferrin,** found in mucosal surfaces, including that of the large intestine. It is an inflammatory marker since it is released by neutrophils at infection sites.
2) **Hemoglobin**, found inside erythrocytes.
3) **Transferrin**, found in serum and lymph. It delivers iron to all cells.
4) **Ferritin,** an abundant protein mainly found in liver, spleen and brain. It is an iron storage protein.

3.1 Lactoferrin and *Entamoeba histolytica:* Between hate and love there are only two iron atoms

3.1.1. HoloLactoferrin as an iron source for *Entamoeba histolytica*

HoloLf (iron-saturated Lf) could be found in abundance at intestinal level during the development and establishment of amoebiasis. This glycoprotein is an **integral part of the innate immune defense** of intestinal mucosa and is normally produced by exocrine glands. In addition, it is released as **apoLf** by neutrophils at the site of infection. In fact, **Lf is an inflammation marker of the colon** [26]

Once apoLf is released either from neutrophils or intestinal cells, the free iron from the environment is sequestered by this iron chelating protein, which is transformed into holoLf, and then it could be used as an iron source needed for the growth, colonization and invasion of pathogens like *E. histolytica*.

Knowing some of the functions of this interesting **moonlighting protein** in other systems, we were interested in the study of the interaction of Lf with *E. histolytica* trophozoites and its effects in amoebae cultured *in vitro*. **We found two opposite effects of Lf** in amoebae when they were cultured in media without iron to which different concentrations of holoLf or apoLf were added:

1) HoloLf is able to provide the iron to support the amoebic growth, and

2) ApoLf is amoebicidal

To know more about this dual effect, by means of which apoLf is able to kill the amoebic trophozoites and holoLf is used as a sole iron source for their multiplication, we developed several experiments: first, when ferric citrate was added to the medium with apoLf, the amoebicidal effect was not observed and amoebic culture is recovered. Our explanation is that **apoLf sequesters the iron from ferric citrate and, once converted into holoLf, this protein is innocuous and contributes to the growth of trophozoites.**

Next, the mechanism of interaction between amoebae and both types of Lf was explored. First, by investigating the interaction between holoLf and *E. histolytica*, we found that **the human Fe-holoLf acquisition mechanism is through a 90 kDa Lf-binding protein (Lbp),** a probable receptor specific for binding to holoLf which is located in the amoebic membrane (**EhLbp**). By kinetics of endocytosis using confocal microscopy, it was found that holoLf is internalized through vesicles coated with cholesterol and a protein **recognized by a mAb directed against mammalian caveolin** [9, 27].

Subsequently, the holoLf carried by these vesicles was delivered to the **endosomal/lysosomal route** in *E. histolytica*. It is known that amoebae contain compartments with cysteine protease activity (lysosomes-like); as Lf was found in these compartments, **it is possible that lysosomal proteases cleave holoLf.** In fact, **proteolytic activities of 250, 100, 40 and 22 kDa against Lf** were found in amoeba extract proteins, all of them were activated at an acidic pH. In addition, **the organelle lysosome-like of amoeba has the acidic environment that could facilitate the iron releasing from holoLf.**

This work described, for the first time, the presence and participation of a protein that is similar to caveolin in *E. histolytica*, and the use of holoLf as a sole iron source for the optimal growth of trophozoites, as well as the cytoplasmic proteolytic activities of *E. histolytica* against holoLf [9]. A recent review that summarizes the use of holoLf as an iron source for parasites is found in: [28].

3.1.2 Amoebicidal activity of apoLf

Lactoferrin adopts different 3-D conformation when it releases the two iron atoms. In the assays with amoeba, apoLf showed amoebicidal activity since the first hours of interaction with the parasite cell and this activity was dependent on the iron absence. **Fe^{2+}, Fe^{3+}, Mg^{2+} and Ca^{2+} protected the amoebae from the amoebicidal effect of apoLf.** This effect was dose-dependent, increased at pH <4.0 and temperature >40 ºC. Regarding the amoebicidal mechanism of apoLf, **some of the target sites could be phospholipids and cholesterol**, both present in the amoebic membrane, since apoLf interacted with them at that level. This interaction caused structural changes such as cellular rounding, vacuolization, damage in the membrane and finally lysis. All of these effects occurred in 1 h, and lipids and apoLf were removed from the amoebae [27, 29].

Currently, ApoLf has been tested as a microbicidal for numerous species of bacteria, fungi and protozoa. A recent review that summarizes the use of apoLf as parasiticidal is found in: [30].

The peptide **Lactoferricin B (Lfcin B)** derived from the N-terminal of bovine Lf, showed similar amoebicidal effect to apoLf against *E. histolytica*; however, the peptide was able to enter in the amoeba cell and **reached the nucleus.** Lfcin B was lethal at a concentration of 1.0 mg/ml, which is a lower dose than apoLf (8.0 mg/ml). Recently, synthetic peptides derived from bovine lactoferrin called **Lactoferrampin** (aa265-284) and **Lactoferricin** (aa17-30), as well as a peptide consisting in the fusion of both peptides by a

lysine, called **Lactoferrin chimera,** were tested in amoebic cultures. All of the peptides assayed were amoebicidal, but the Lf chimera showed the major amoebicidal effect [60].

We also found that **apoLf apparently acts in synergism with IgA and lysozyme** from human milk, since when the three proteins were incubated with amoebae at a concentration of 1 mg/ml each, the lethal effect was observed in only 30 min of incubation. In addition, a quarter of **metronidazole** concentration (2.5 µg/ml) used to kill amoebae *in vitro* plus a quarter of apoLf (2.5 mg/ml), were able to inhibit the growth *in vitro* of *E. histolytica*, suggesting **a synergistic effect** [29].

Interestingly, data **using only apoLf in amoebic liver abscesses induced in hamsters show that animals were cured [31].** Also, in **a murine model of intestinal amoebiasis,** apoLf was able to reduce lesions of the **caecum** and cure the amoebiasis [32].

In summary, holoLf is recognized by a binding protein located on the surface of amoeba, then Lf is endocytosed through caveola-like vesicles, and it traffics through endosomes and lysosomes, where the iron is probably released from holoLf. In lysosomes the protein is degraded, and the metal is used by the amoeba to maintain growth. On the other hand, apoLf binds to amoeba phospholipids and cholesterol but afterwards, Lf is released and this leads to lysis. The effectiveness of apoLf, either alone or in concert with other components of milk or metronidazole, is evident as an anti-amoebic, and this is reinforced by results in animal models.

We can conclude about the curious bipolar love-hate relationship among amoebae and Lf, which only depends on the two iron atoms present in the environment where amoebae reside to have an effect in the life of this parasite. This is a classic example of why Lf is considered as a "**frenemy**" protein (friend and enemy).

3.2 Hemoglobin: the erythrocyte is the bread and wine for *E. histolytica*

3.2.1. Hemoglobin as an iron source

More than 100 years ago, **Fedor Aleksandrovitch Lösh** demonstrated **erythrocytes or red blood cells (RBCs) inside amoebae in fecal samples** of a Russian patient suffering amoebiasis [33]. Since then, much information has been accumulated regarding the relationship between erythrophagocytosis and *Entamoeba* virulence. **The RBCs are phagocytosed and stored in amoebic vacuoles and then destroyed in order to utilize their content.**

In fact, erythrophagocytosis is considered by some researchers as an important criterion to evaluate the virulence of different species of *Entamoeba* [34, 35]. However, other authors did not find the relationship between erythrophagocytosis and virulence [36]. *E. histolytica* trophozoites have also a peculiar process of endocytosis of Hemoglobin characterized by **random suction of the RBC content** [37] (Figure 1).

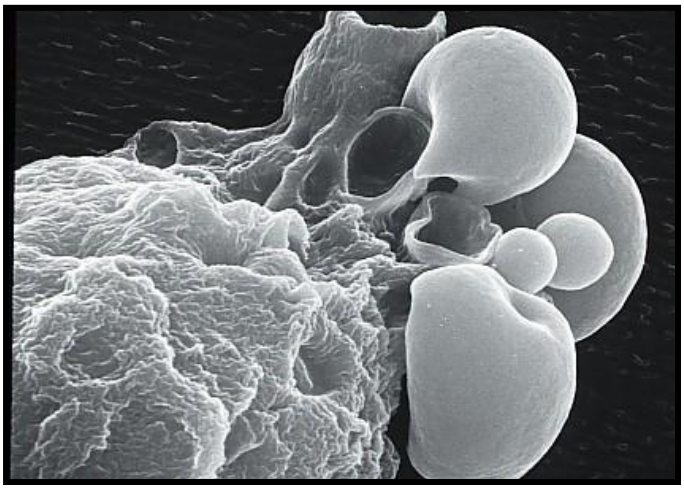

Figure 1. SCANNING ELECTRON MICROSCOPY: *Entamoeba histolytica* trophozoites in the process of phagocytosis of human erythrocytes.

Courtesy of Dr. Victor Tsutsumi. Department of Infectomics and Molecular Pathogenesis, CINVESTAV-IPN, México.

Trophozoites can phagocytose up to 40 RBCs/amoeba/h [38] and digest them inside vacuoles. The half lifetime of RBC inside the vacuoles is about 2 h [39] and during the next 9 h the Hemoglobin (Hb) is completely degraded [40].

Several researchers have studied the role of amoebic **Hemoglobinases** in degrading Hemoglobin from different origin. Jarumilinta and Maegraith [41] observed the degradation of native bovine Hb at pH 6.0 by amoebic crude-extract proteases from different monoxenic strains (cultured with a bacterial species).

McLaughlin and Faubert [42] purified two proteinases that are able to degrade native bovine Hb, one of 41 kDa with optimal activity at pH 3.5 and another of 27 kDa with optimal activity of pH 6.0. Lushbaugh et al in 1984 [43] described the presence of a **Cytotoxin of 22 kDa with strong activity against denatured Hb.** One year later the same authors [44] were able to purify a **Cathepsin B of 16 kDa that was active against native and denatured bovine Hb.**

Pérez-Montfort and his group [45], by using **substrate-gel electrophoresis** showed the presence of two proteases of 32 and 40 kDa that were able to degrade denatured Hb. In 1998, our group described three proteases of 21, 82, and 116 kDa in crude extracts of *E. histolytica* HM1:IMSS strain. These proteases belong to **Cysteine-proteinases and cleave human, bovine, and porcine Hb** mainly at pH 7.0, their activity is maintained in parasites cultured either in the presence or absence of iron in the culture medium. In our group

we also demonstrated that Hb is an excellent iron source for amoeba´s growth [46] (Figure 2). Becker and her collaborators [47] reported an **active cysteine-protease of 30 kDa in vacuoles that previously contained phagocytosed RBCs**. Recently, two Hb binding proteins have been described: one of 45 kDa **(*Eh*hmbpd45)** [49] and another one of 26 kDa **(*Eh*hmbpd26)** [50]; both proteins are over-regulated by the absence of iron or the presence of Hb.

One way used by *E. histolytica* trophozoites to obtain Hb is through the disruption of RBCs by Hemolysins [48].

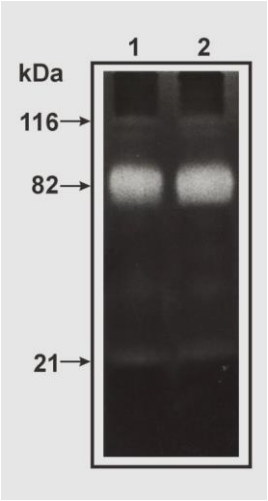

Figure 2. *Entamoeba histolytica* HEMOGLOBINASES.
Lane 1, trophozoites growth in the presence of iron
Lane 2, trophozoites growth in the absence of iron

It can be concluded that *E. histolytica* trophozoites could utilize human Hb as an iron source as soon as they invade the large intestine and during their travel to the liver; in this organ, Hb is used as a nutrient thus contributing to the virulence and lesions caused by the parasite.

3.3. Transferrin: iron transport hacked by amoeba

3.3.1. Transferrin as an iron source

Iron-charged Transferrin can be used as an iron source by pathogens when they have access to the blood and other fluids. In fact, some bacterial and parasitic species possess specific Transferrin receptors (TfRs) depending on the animal species that they infect. A recent review that summarizes the use of holoTf as iron source by parasitic protozoa is found in [51].

E. histolytica, the parasitic protozoan that causes amoebiasis, **has the ability to bind human holoTf to satisfy its need for iron;** this protozoan has been poorly studied in this aspect. Trophozoites are able to survive in culture medium treated with Chelex-100 (to remove most of the iron contained), to which 1.4

mg/ml of holoTf are added, providing a final concentration of approximately 60 µM of Fe [52]. HoloTf binds to at least two proteins present in extracts obtained from trophozoites, of 70 and 140 kDa. One of them (140 kDa), interestingly, is recognized by the anti-human Tf receptor B3/25 (TfR1) mAb; as this antibody is heterologous, **the amoeba holoTf binding protein could only be structurally similar to the human TfR1.**

3.3.2. HoloTf endocytosis

When the mentioned mAb is used, the **internalization of holoTf, together with its binding protein** takes place in a maximum time of 30 min [52]. Furthermore, **the protein that recognizes holoTf is located on the amoeba cytoplasmic membrane** (Figure 3). When the cell is incubated with holoTf allowing it to internalize this ferric protein, both the protein recognized by the mAb and holoTf are internalized, probably by forming a complex. **This mAb recognizes a protein that binds holoTf (EhTfbp) which is involved in its internalization.**

The **internalization of holoTf together the Tf-binding protein** starts at very short times; in 1-2 minutes the complex is inside the trophozoite. Because this endocytic process is very fast, probably it is a **constitutive mechanism**, i.e. not activated when the ligand binds to the binding protein, but remains active all time, unlike what is observed in internalization of holoLf, where it is activated once the receptor binds to its ligand [9]. The internalization was followed up to a maximum time of 45 min. At this time most of holoTf was inside the trophozoite (Figure 4). These experiments **reveal the presence of a protein that recognizes holoTf;** additionally, we have found that **amoeba is able to discriminate holoTf from apoTf,** as the latter was not bound or internalized.

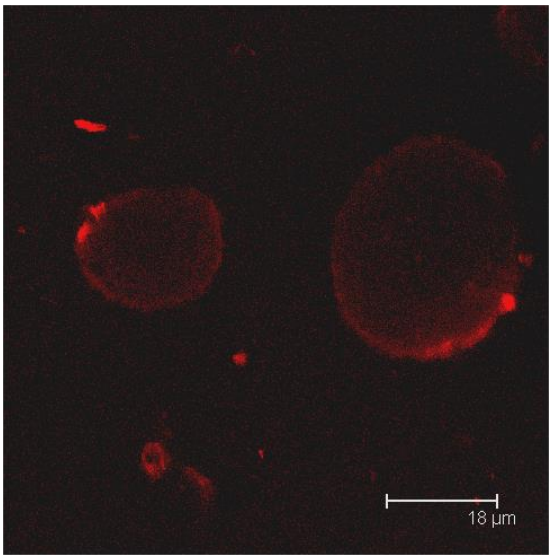

Figure 3. ANTIBODY RECOGNITION OF ANTI-HUMAN TRANSFERRIN RECEPTOR IN *Entamoeba histolytica* TROPHOZOITES.

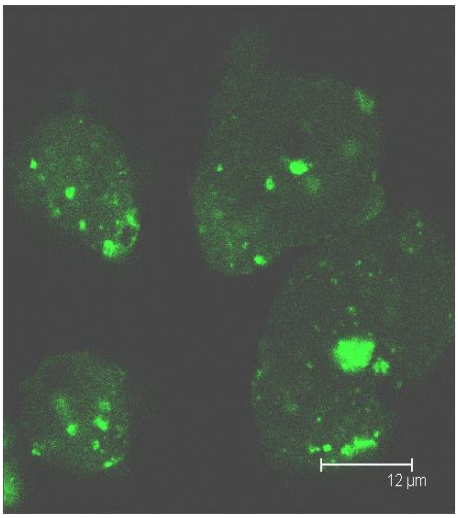

Figure 4. INTERNALIZATION OF FLUORESCEIN-LABELED TRANSFERRIN, 30 min of incubation.

Other authors reported that the holoTf internalization appears to be receptor-independent [53]. Therefore, *E. histolytica* has two ways to internalize holoTf, one through an EhTfbp, which is specific for holoTf and recognizes this ligand, and another one independent of receptor, in which holoTf continuously is entering to the cell without reaching saturation. This is explained by the great need for this element by the parasite, **so it has evolved at least two mechanisms to obtain iron from human holoTf,** with different affinity, depending on growing conditions, as is the case of most organisms with two or more iron acquisition systems.

During the amoebiasis course, this parasite faces several molecules containing iron that can be used for its growth and spreading. The capacity to use multiple iron supplies allows it to travel and settle in various host tissues. The presence of several iron acquisition systems gives an advantage to the trophozoites. Likewise, the presence of two mechanisms for obtaining iron from holoTf may be beneficial for the development of infection.

To discern about the process of holoTf internalization, we studied distinct routes of endocytosis. Trophozoites were treated with **chloroquine**, an inhibitor of endocytosis through clathrin-coated vesicles in receptor-mediated endocytosis. This compound specifically **modifies the pH of endocytic vesicles, to avoiding their separation from the cytoplasmic membrane.** In *E. histolytica*, the internalization process was completely inhibited by chloroquine, this is evidence that holoTf binds to a Tfbp in the cytoplasmic membrane and then interacts with clathrin-coated vesicles (Figure 5).

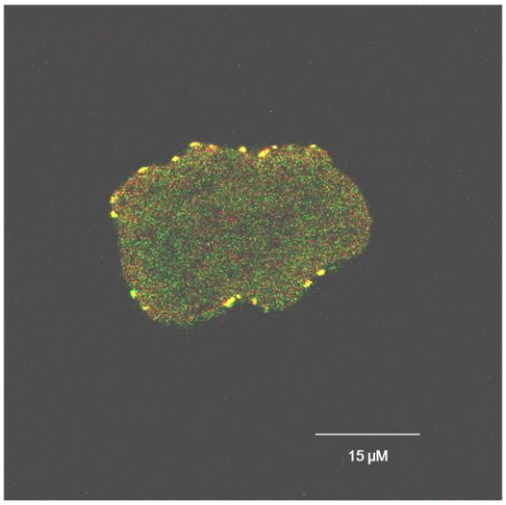

Figure 5. LOCALIZATION OF CLATHRIN USING AN ANTI-BOVINE BRAIN CLATHRIN Ab (red) IN TROPHOZOITES OF *E. histolytica* INCUBATED WITH FITC-Tf (green).

The co-localization is shown in yellow.

The endocytosis process via clathrin involves the actin cytoskeleton [54, 55], so depolymerization of actin in trophozoites reduces the holoTf entry by almost 90%. This indicates that **amoeba requires an intact cytoskeleton to carry out endocytosis of holoTf.** In a recent paper we demonstrate that the enzyme **Acetaldehyde/alcohol dehydrogenase 2 (ADH2)** is also involved in the holoTf endocytosis [56]. Apparently, the mechanism of iron obtaining in *E. histolytica* is composed by a protein complex that requires further study to fully understand the process.

3.4. Ferritin: Amoeba knows the combination of the host safe box that stores iron

3.4.1. Ferritin as an iron source

Research about the use of Ferritin as an iron source by pathogens is limited. A recent review summarizes the works published up to date [57].

***E. histolytica* is a versatile microorganism able to parasitize several organs in the human body, mainly the large intestine and liver**. In both organs amoebae produce **abscesses, which can cause the death**.

Ferritin is mainly found in the liver hepatocytes; this organ contains up to 1 g of Ferritin, with the **capacity to store 50% of the iron corporal reserves**. Therefore, we can assume that in **the invasion process by *E. histolytica*, Ferritin could be an important iron source** for this parasite.

In the liver, amoeba could utilize Hb from erythrocytes as an iron source; however, **Ferritin would provide to the parasite 1,000 times more iron than Hb!!!** Also, the use of Ferritin could be associated with the parasite virulence, probably due to the efficiency of its degradation. In this work we studied whether Ferritin is an important iron source for amoeba. This is a first step for deciphering the colonization process in the liver by *E. histolytica*, in order to study the invasion process.

***E. histolytica* is able to use Ferritin as a sole iron source** in axenic culture medium without ferric citrate, after the iron traces are removed with the resin Chelex-100. We showed that the optimal concentration for growing is **100 µM of iron**, **supplied by Ferritin.** A lower or higher concentration than this value leads to decrease of the optimal growth of amoebae. **Amoeba proteases are able to hydrolyze Ferritin in several fragments** of different molecular weight, when the amoebic total extract is analyzed in substrate gels by electrophoresis in polyacrylamide co-polymerized with Ferritin. **These proteases are of cysteine type, and they are active at neutral and acidic pH.** The result of the interaction amoeba-Ferritin is found in the reference [55].

3.4.2. Binding, endocytosis and traffic of vesicles containing Ferritin

Trophozoites of *E. histolytica* are capable of binding Ferritin. This protein was labeled with fluorescent isothiocyanate (FITC-Ferritin), and it was observed by **confocal microscopy like patches in the amoebic surface before 15 seconds of incubation**. This result suggests that amoeba possesses a **Ferritin binding protein (Ftbp) or receptor on its surface**, which participates in the process of binding to this protein.

At subsequent times (but from the first minute), we found FITC-Ferritin in cytosolic vesicles, maintaining this behavior until 60 min. This same result was observed by electronic microscopy using **cationized Ferritin,** since this protein is electron-dense due to its high iron content. This experiment shows that amoeba internalizes Ferritin and that probably is through a Ftbp. This protein is internalized by amoeba with its iron content, discarding the possibility of the existence of a ferric reductase in surface [55].

The purpose of this study was to analyze the internalization of Ferritin by amoeba. Experiments were carried out first by **kinetics of binding with this protein, which were evaluated by flow cytometry for different times and temperatures**. At 37 °C and since the first minute, a considerable increase in the fluorescence intensity was observed, reaching to its maximum intensity at 5 min and the value was maintained for 90 min; this is the normal behavior of a typical endocytosis, indicating saturation with respect to time. At 4 °C we observed an evident decrease of fluorescence intensity, approximately of 80%. With this result we suggest the following:

1.- **Amoeba internalizes Ferritin.**

2.- Amoeba **possesses a Ferritin-binding protein (EhFtbp).** This molecule participates in reaching the prolonged saturation and avoids the indiscriminate internalization of Ferritin to the cell.

3.- **EhFtbp participates in the Ferritin internalization**. Previously, it has been described that at 4 °C the endocytic processes mediated by receptor and all of the energy dependent transports are stopped. In base of these results we suggest that this process is dependent on time and temperature and this event could be regulated by a Ferritin binding protein.

Furthermore, it was shown that ***E. histolytica*** **internalizes Ferritin by a process of endocytosis via clathrin-coated vesicles.** Endocytosis of Ferritin was analyzed using different inhibitors of endocytosis. **Wortmannin** (inhibitor of the liquid phase) and **Filipin** (caveola inhibitor) did not block the internalization of Ferritin, this occurred only using inhibitors of clathrin, such as **chloroquine, NH_4Cl, sucrose, and chloropromazin**e [58]**.**

To test the involvement of clathrin in endocytosis of Ferritin, it was conducted a trial of co-localization of clathrin-Ferritin. In 2 minutes of incubation with clathrin-FITC and Ferritin, it was found co-localization of both proteins; in this case amoebic clathrin was identified with an anti-clathrin from bovine brain by confocal microscopy. Furthermore, the presence of clathrin-coated vesicles which contain cationized Ferritin were observed by electron microscopy [55]. The results suggest that:

1. **Clathrin from a primitive protozoan such as *E. histolytica* possesses a similar epitope to mammalian clathrin**.

2. **Ferritin is endocytosed by means of clathrin-coated vesicles by amoeba,** a similar process such as occurs in other mammalians cells;

3. **Ferritin internalization is practically immediate after interaction with the amoebic surface, a probably constitutive mechanism**, although this should be confirmed. On the other hand, our results show that Ferritin internalization by amoeba was of 70% by clathrin coated vesicles and the 30% remainder was carried out by an unknown process.

Finally, the **traffic of Ferritin in vesicles was studied**. Ferritin co-localized with a classic lysosome marker, **LAMP-2**. In this case we used an anti-human LAMP-2 antibody. The results suggest that *E. histolytica* **shows an endocytosis process similar to that of high eukaryotic cells**. Sequence of the amoebic protein similar to LAMP-2 is unknown, but it will be very interesting to investigate the degree of homology/identity that it presents in comparison with that of mammalian cells, to know its evolution and possible origin.

By means of **Western blot and overlay assays with peroxidase-Ferritin (HRP-Ferritin),** this protein recognizes two amebic proteins of 135 and 140 kDa, which show specificity to Ferritin. The possible function of these proteins in Ferritin endocytosis could be to recognize Ferritin in amoebic surface and probably send a signal for trigger the endocytic process of Ferritin for finally using this protein as an iron and amino acid sources.

In addition, **the amoebic surface was saturable by Ferritin** since this protein was bound in a concentration-dependent manner. Amoebae reached a maximum saturation with 4.6 x 10^{-8} M of FITC-Ferritin, suggesting that **the binding protein (Ftbp) has a high affinity to Ferritin.** This result can suggest a regulation in the binding process and probably in Ferritin endocytosis.

Currently, we can suggest the possible binding and internalization mechanism of Ferritin in *E. histolytica*:

Amoeba recognizes Ferritin through an Ftbp, which can rapidly bind specifically with high affinity and probably regulate internalization, because the binding is concentration-dependent and saturable. The interaction Ferritin-Ftbp is a process mediated by clathrin-coated vesicles. Then, Ferritin is transported to the early endosome, probably in this compartment begins the process of degradation of Ferritin because it was found that amoebic cysteine proteases active at neutral pH were specific for this protein. Later on, Ferritin was found in late endosomes and finally lysosomes, where this ferric protein is completely hydrolyzed; in this compartment there are cysteine proteases active at acidic pH which could cleave Ferritin.

Once Ferritin is degraded by the amoeba, the parasite uses Ferritin iron for the synthesis of important molecules of essential metabolism, such as several Fe-S proteins, SOD, etc. It is important to note that we not rule out the presence of a molecule warehousing of iron (amoebic Ferritin) to safeguard this element.

There are few studies in the literature on the mechanisms of liver invasion by amoebae. A possible mechanism could be: amoebae parasitizing the colon travel through the portal vein reaching the liver. It is not known if this body organ has a molecule recognized by amoeba, or if the amoeba only reaches the liver as the first organ. In the liver, amoebae release several molecules such as cytolysins that lyse hepatocytes. Then, the lysed hepatocytes release their contents, where Ferritin is in large iron quantity, since it contains abundant iron atoms. Finally, amoebae internalize Ferritin and use this protein as a source of iron in the process of colonization and the formation of amoebic liver abscesses.

In summary, in Figure 6 we show a cartoon of the findings about the use as an iron source and endocytosis of the four host iron-containing proteins aforementioned.

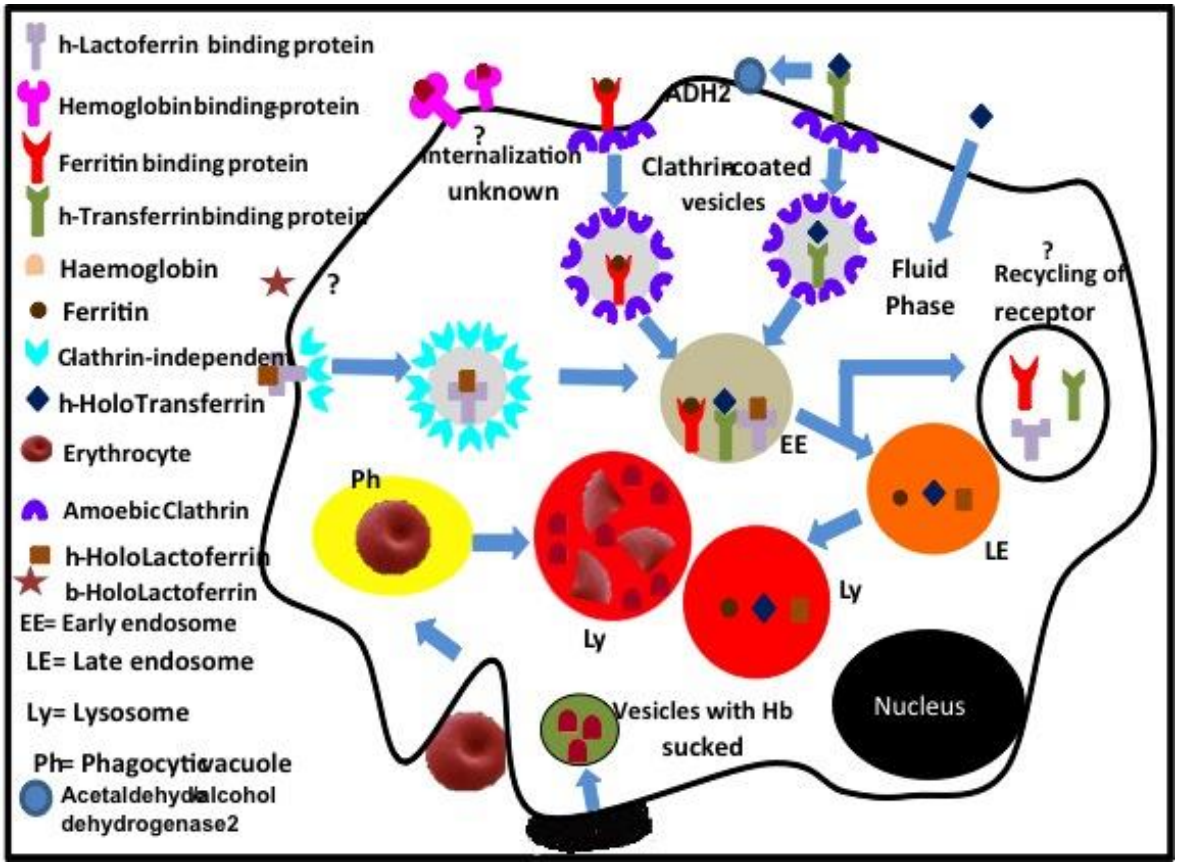

Figure 6. MECHANISMS PROPOSED FOR ENDOCYTOSIS AND DEGRADATION OF SEVERAL IRON SOURCES BY *Entamoeba histolytica* HM1:IMSS. (human, h; b, bovine).

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Chapter 18

The Ironic *Leishmania*

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Abstract

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Abstract

Leishmania species are trypanosomatid parasites which have a biphasic life cycle, the promastigote form in the invertebrate vector, and the amastigote in the vertebrate host. These parasites are responsible for zoonotic and human diseases with a high index of morbidity and mortality worldwide.

Iron is an essential nutrient for most organisms, and its metabolism is regulated and secured to prevent that pathogens can make use of it. When iron bound to Transferrin is phagocytosed through the endocytic pathway by the Transferrin Receptor (TfR1), the acid conditions in late endosomes dissociates iron from Transferrin. Then, iron is converted by endosomal reductases from Fe^{3+} to Fe^{2+}, and exported by transporters such as DMT1 proteins and NRAMP-1 and -2 to cytosol, where it is stored by Ferritin.

However, *Leishmania* captures the Fe^{2+} before it is exported from phagolysosomes or late endosomes, in parasitophorous vacuoles (PV) were amastigotes reside intracellularly, for its use in iron metabolism, with the help of reductases and iron transporting proteins bound to the parasite membrane, which are similar to the transporters of the host.

The hit of *Leishmania* in the iron acquisition is to live inside of the organelle where iron security pathway takes place.

Key words: *Leishmania*, iron, LIT1, LIT2, ZIP.

1. Introduction

The genus *Leishmania* corresponds to **trypanosomatid parasites** that infect human beings and other mammals. In the invertebrate host, the **sand fly from the genera *Phlebotomus* and *Lutzomyia*,** parasites proliferate as **promastigotes (flagellated form),** first in the replicative, non-infective **procyclic phase.** Afterwards, they transform in the infective, replicative **metacyclic phase,** in the insect intestine.

In the mammalian host, the amastigotes are the only parasites that proliferate; they are obligate intracellular parasites (non-motile) that infect mainly mononuclear phagocytes [1]. Around two million new cases occur every year (1.5 million of cutaneous leishmaniosis and 0.5 million cases of visceral leishmaniosis), with an estimate of **12 million people infected around the world.**

There are 5 main species of *Leishmania* that can infect humans: *L. tropica, L. major, L. donovani, L. braziliensis* and *L. mexicana*; the disease they produce can be classified in 3 different clinical forms: *cutaneous (CL), visceral (VL) and mucocutaneous (MCL) leishmaniosis*. The type and severity of the disease depend on the species, genotype and nutritional condition of the host, the vector, and some social and ambient factors [2].

When promastigotes are inoculated in the mammalian host dermis, parasites are phagocytosed by macrophages, where they transform into **the amastigote form within an organelle rounded by a membrane, that belongs to the endocytic route, and that progressively acquires characteristics of a late endosome.** The compartments hosting mature parasites are known as **parasitophorous vacuoles (PVs)**, and their morphology varies depending on the *Leishmania* species.

The **PVs are acidic compartments** that contain several **lysosomal enzymes**. These vacuoles are surrounded by a membrane, enriched with proteins from late endosomes and lysosomes, such as **Rab7p**, **Macrosialin**, **Lamp-1** and **Lamp-2,** and **vacuolar ATPase H^+**, as well as molecules from the antigen presentation machinery, such as **class-II MHC and H-2M** in macrophages treated with γ-IFN [3, 4].

Both, amastigote phagosomes or metacyclic-promastigote phagosomes fuse with late lysosomes around 30 min after cell penetration. However, in some species the formation of PVs with amastigotes takes longer times, in comparison with metacyclic promastigotes, as with *L. amazonensis*. This indicates that the **fusion process is dependent on a series of events that are species-, and most probably stage-specific** [4].

***Leishmania* coexists and spreads in two different environments, which differ mainly in their acid-base properties.** The pH in the vector medial intestine is around 7.0-9.0, and in the mammal' phagolysosomes of macrophages is 4.5-5.5. Adaptation of the parasite to different environmental conditions demands regulatory mechanisms to maintain the cellular function. These mechanisms also need to adapt to acidic charges generated through the **anaerobic metabolism** of the parasites. **A bicarbonate transport**

system is responsible for the regulation of the pH homeostasis in the *Leishmania* promastigotes. The main anionic pathway in this parasite is sensitive to anion transport blockers and is apparently conductive in nature and provides ions such as bicarbonate and chloride.

Under physiological conditions, the main role for the proton pump is the production of a relatively long membrane potential that functions as a trap for electrochemical nutrients. **The natural environment of the parasite, both alkaline and rich in HCO_3^-/CO_3^{2-}, Cl^-, HCO_3^- or the exchange Cl^--OH^- constitutes the primary mechanism of regulation of cellular acidification or alkalinization in response to pH changes** [5, 6].

2. Homeostasis of Iron

Iron is an **essential nutrient** for most living organisms. Its importance relays in the **flexibility of the redox potential** due to a variety of interactions with ligands, electrons transference and acid-base reactions. Iron is also a co-factor of a great variety of proteins that can be classified according to the chemistry of the metal:

1) **Heme-proteins**, which serve as carriers of O_2 or electron transporters;

2) **Iron-sulfur coupled (ISC)** proteins, mainly involved in electron transference;

3) Proteins that only contain iron, including **enzymes and those involved in iron transport and storing** [7].

The free iron concentration in blood and extracellular fluids is null virtually (10^{-18} M). In plasma, circulating iron is bound to **Transferrin (Tf)**, whereas in secretions such as colostrum, milk and mucosal secretions iron is bound to **Lactoferrin (Lf)**. Both are glycoproteins with two iron binding sites for Fe^{3+}, the ferric form of iron bound in aerobic environments.

At neutral pH, capture of diferric-Tf is mediated by the **Transferrin Receptor-1 (TfR1),** which facilitates the internalization of the Tf-TfR1 complex through endocytosis. Inside early endosomes, iron is released after reduction of the pH to 5.5 by a proton pump, provoking the separation between iron and Tf. The metal ion is then transported, via the **Divalent Metal-ions Transporter (DMT-1),** through the endosomal membrane pathway [8] (Figure 1).

Iron metabolism is carefully regulated and secured by iron regulatory proteins. These molecules bind to **Iron Response Elements (IREs)** in the **mRNA** that will be translated into proteins involved in: 1) iron capture **(TfR1 and DMT-1)**, 2) iron usage **(D-amino-Levulinic erythroid acid synthase)**, 3) iron storage **(Ferritin)** and 4) iron export **(Ferroportin-1)**. Another key component in the regulation of systemic iron homeostasis is the peptide **Hepcidin,** which regulates cellular iron circulation by binding to **Ferroportin-1** and inducing its internalization [9].

Pathogenic microorganisms use different strategies for iron capture from the host. Therefore they need to compete efficiently for the iron-protein complexes, because this element is very important for their survival inside the host cell. A great variety of bacteria acquire iron by the **secretion of siderophores** [10], low molecular weight compounds with high affinity for iron, that compete efficiently with the host proteins that bind iron.

Moreover, alternative mechanisms for iron acquisition have been reported in bacteria, such as the presence of **receptors that bind iron-binding proteins** such as Lactoferrin and Transferrin [11], **proteases that degrade iron-binding proteins** to release the metal [12], and **reductases that reduce the ferric iron.** In this way, pathogens can also release the metal from protein complexes (Lactoferrin and Transferrin) [13].

Currently, there is no evidence that protozoa can acquire iron through siderophores and siderophores receptors. A study with *Leishmania chagasi* showed that the known parasite-secreted soluble molecules are unable to remove iron from Lactoferrin or Transferrin. **Exogenously added Tf is able to reach intracellular parasites and promote their growth;** however, it is not yet clear whether enough Transferrin-bound Fe^{3+} is available under physiological conditions [14].

3. Iron and Trypanosomatids

Studies on susceptibility and genetic predisposition for disease development in mammalian hosts and pathogens' virulence determinants have identified iron as a key point. **Both pathogens and their hosts have developed iron procurement processes and iron chelating systems that function oppositely to withdraw this metal in the phagosome environment**. In this way, iron is a key element during the macrophage-parasite interphase [15].

In trypanosomatids, acquisition of Transferrin-chelated iron has been demonstrated only for the African trypanosomes such as *Trypanosoma brucei* [16]. These observations agree with the fact that **African trypanosomes are exclusively extracellular in their mammalian host and in this way they are exposed directly to holoTf, the more abundant source of iron in serum** [17, 18].

On the other hand, it is not really clear **why a receptor-mediated pathway to capture Tf, is available in two trypanosomatid parasites with life cycles that include intracellular stages, *Trypanosoma cruzi* and *Leishmania*** [19]. After entering the cell in the lysosomal vacuole, *T. cruzi* escapes towards the cytosol and replicates in direct contact with the cytosolic Fe-storing protein **Ferritin**. In contrast, *Leishmania* maintains its life cycle inside the endosomal route, where one of the main sources of iron is Fe^{3+} bound to Tf, or Fe^{2+} generated from Fe^{3+} by **endosomal reductases** [20].

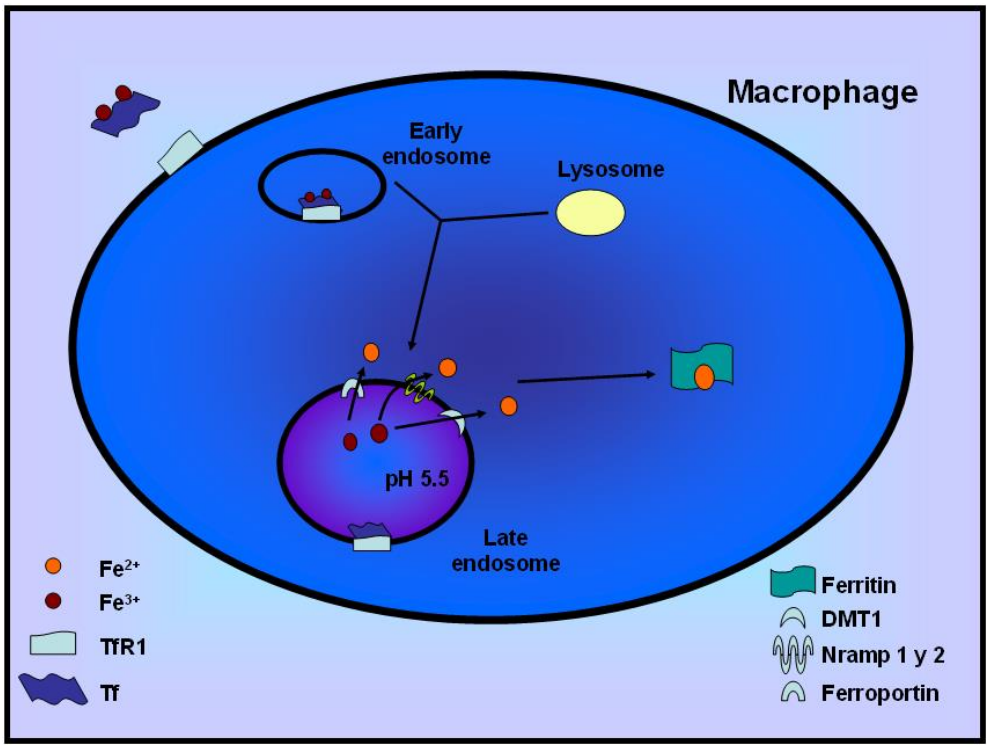

Figure 1. IRON HOMEOSTASIS.

Transferrin (Tf) with two iron molecules bound is phagocytosed through the endocytic pathway by the Transferrin Receptor (TfR1). Once it reaches late endosomes, the acidic micro-environment dissociates iron from Tf and converts Fe^{3+} to Fe^{2+} through endosomal reductases; afterwards Fe^{2+} is exported by transporters like DMT1 and Nramp 1 and 2 proteins to the cytosol, where it is stored by Ferritin.

4. Iron acquisition by *Leishmania*

The parasitic protozoan *Leishmania* has developed different **adaptation systems** that have allowed it to survive within the host cell, that to date are not completely understood. It is well known that **intracellular survival of parasites produces pleiotropic effects in macrophages**, because the parasite adversely affects innate immune functions of these cells. To this respect, we can mention: **inhibition of the respiratory burst, prevention of apoptosis, inability to present antigens, diminished response towards cytokines, chemotaxis inhibition in both macrophages and neutrophils, and suppression of the Th1-type protective response** [21].

Iron uptake by *Leishmania* depends on a complex system that involves several factors, among them, **receptors, ferric reductases and iron transporters**. One of the best characterized **susceptibility determinants for the infection** with *Leishmania major* in the murine model is the Nramp1 **(NRAMP1 or SLC11a1)** protein. Mutations in *NRAMP-1* gene are responsible of susceptibility to the infection with intracellular parasites such as *L. donovani*, *Salmonella* and *Mycobacterium*.

NRAMP1 is located in lysosomal compartments inside phagocytic mononuclear cells and also in neutrophils granules and it is rapidly recruited towards the membrane of phagosomes that contain phagocytosed microorganisms. In the phagosome membrane, **NRAMP1** functions as a proton-dependent pump that removes **Fe^{2+} and Mn^{2+} ions in the intraphagosomal environment.**

NRAMP1 chelating of metallic ions restricts **bacterial multiplication and activation of macrophages**. As well, it has been found that **NRAMP1** participates in *Leishmania* replication. In this way, **NRAMP1** belongs to a group of **iron biological chelants** that are present in macrophages, including **NRAMP2 (SLC11a2), Ferroportin (SLC40A1) and Lipocalins**, all of them representing the initial phase of defense of cells against pathogens invasion [22].

In the endosomal pathway of macrophages, there is a low level of Fe^{2+} due to the presence of NRAMP1 and **NRAMP2**; therefore, inside the PVs this metal is found in small quantities and then its use by the parasite is minimal. One of the most important findings to understand the capture mechanisms of iron by *L. chagasi* is **the preferential intake of iron in its reduced form (Fe^{2+}).** This finding showed that *L. chagasi* expresses a **NADPH-dependent iron reductase** able to convert Fe^{3+} to the soluble Fe^{2+} form [13] (Figure 2).

The presence of **Ferric reductases** has been reported in the *L. major*, *L. infantum* and *L. braziliensis* genomes, and they have been named as LmjF30.1610, LinJ30_V3.1630 and LbrM30_V2.1670, respectively [23]. **The predicted protein shows a significant homology with the Fe^{3+} chelate reductase of *Arabidopsis thaliana*** [24].

Similarly, it has been described that *Leishmania* expresses iron transporters that belong to the **ZIP family**. Members of this family contain eight transmembrane domains with amino- and carboxyl-terminal sequences located in the extracellular side of the plasma membrane [25, 26]. Two identical genetic sequences have been found, the LmjF31.3060 and the Lmj31.3070 (LIT1-1 and LIT1-2) in the genome of *L. major*. These sequences have a 30% identity and 53.8% similarity with the **IRT1 transporter of *Arabidopsis*. This may reflect the ability of *Leishmania* to compete with the transporters present in the endocytic route in the host cells.** The predicted protein encoded by these genes was denominated **LIT1** in *Leishmania*; it presents a **similarity in the membrane topology with IRT1 from *Arabidopsis*** [27]. This region showed to be conserved among the members of the ZIP family; moreover, LIT1 protein also contains an **H×H×H sequence** that has been proposed as **the metal binding domain** [28, 29].

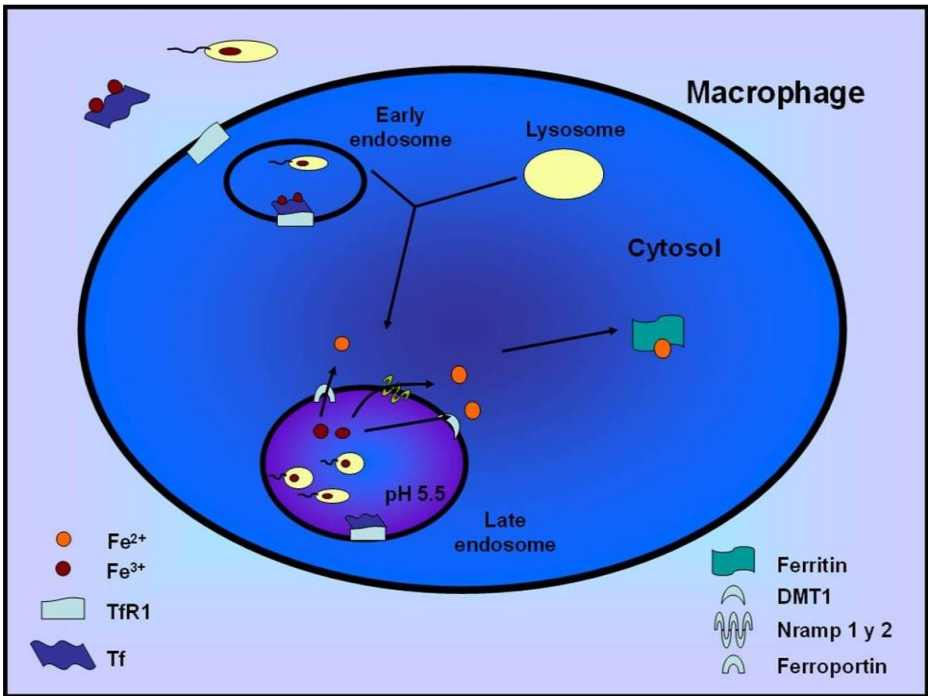

Figure 2. ENDOCYTIC ROUTE DURING PHAGOCYTOSIS OF *Leishmania*.

When *Leishmania promastigotes* are phagocytosed by macrophages, they are internalized via early endosomes where later on, they transform into the amastigote form. Then lysosomes fuse with early endosomes to originate late endosomes, known as parasitophorous vacuoles (PVs); in this microenvironment there is competition for iron between macrophage transporters and transporters expressed on the surface membrane of the amastigotes.

In complementation and ^{55}Fe direct incorporation assays in *L. amazonensis*, it was demonstrated that **LIT1 protein is a divalent metal transporter** with preference for iron localized in the parasite plasma membrane, although this protein has been detected only in amastigotes inside PVs of infected macrophages.

This selectivity in the expression of the transporter suggests that LIT1 may be over-regulated by minimal iron concentrations. These observations have been supported by studies done in **NRAMP1**+/+ **macrophages where the expression of LIT1 is accelerated during amastigotes proliferation,** finding very low levels of intra-phagosome iron in these infected cells in comparison with the iron level in **NRAMP1**−/− macrophages [27]. Therefore, **it is suggested that the LIT1 transporter may be over-regulated by iron restriction, as has been reported in *Arabidopsis*** [30, 31] (Figure 3).

On the other hand, it has been determined that **LIT1 is required for the development of amastigotes and promastigotes in axenic cultures of *L. amazonensis*,** suggesting the existence of **facultative mechanisms for iron acquisition of the extracellular forms** of the parasite [32].

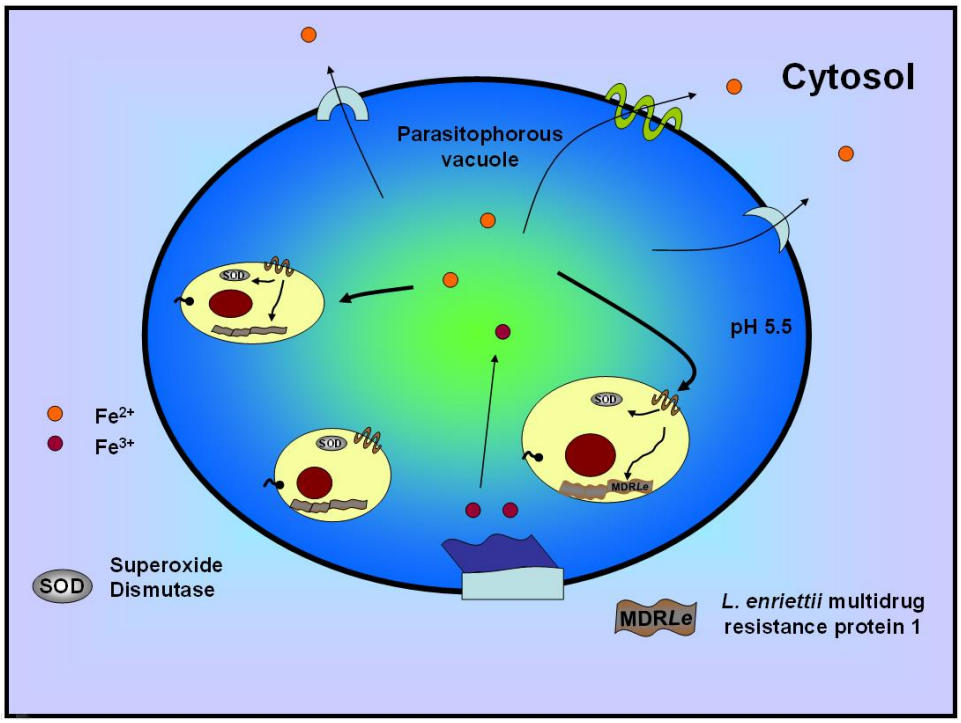

Figure 3. CAPTURE OF IRON IN THE PARASITOPHOROUS VACUOLE.

When parasites are inside of the parasitophorous vacuoles (PVs), they have access to Fe^{3+} released from the Tf:TfR complex; it is then converted to Fe^{2+} and exported to the cytosol; it is then when the parasite competes for iron to internalize it through host analogue transporters which are over-expressed in the presence of reduced iron concentration; once internalized, iron is utilized by the parasite for different purposes such as proliferation and survival.

This hypothesis is based on the existence of the LmjF28.1330 and/or LmjF33.3200 gene products that encode for ZIP-permease domains. The function of LIT1 was determined by its participation as a virulence factor of *L. amazonensis*, when mutants lacking the genes corresponding to this protein were used (LIT1-/-). The mutant strain was able to develop and differentiate in axenic culture; however, it presented a reduced capacity of replication inside the macrophage PVs and induced less cutaneous lesions in mice. **Microsomal genic complementation** reconstitutes the pathogenic process in this parasite. Another and interesting observation is the persistence of a considerable amount of parasites for several months without causing tissue damage in the mouse [27].

5. Participation of iron in the biology of *Leishmania*

Iron is required by *Leishmania* for some functions of its cellular metabolism, among them, the **antioxidant function of the Superoxide Dismutase (SOD)** and some other essential specific reactions of the parasite. The relevance of SOD for this parasite relies in its role as a buffer for the oxygen reactive intermediates during the oxygen burst; this metallo-enzyme **converts O^{2-} into H_2O_2 and H_2O**. There are two SOD genes, *SODA* and *SODB*, which possess conserved sequences for the SODs that use iron as an essential cofactor, and this enzyme was originally identified in *L. chagasi*. **Overexpression of these genes in *L. tropica*, protected the parasites from free radicals produced by nitroprusiate and paraquat** [33].

Another detoxifying heme-containing enzyme in *Leishmania* parasites is the **Ascorbate peroxidase** from *Leishmania major* (**LmAPX**), capable of detoxifying hydrogen peroxide. LmAPX is a heme-containing peroxidase of the parasite and resides in the intermembrane space of the mitochondrial inner membrane in *Leishmania* with a form <3.6 kDa shorter than that encoded by the full-length gene. It is also important for H_2O_2 detoxification and **protection of cardiolipin oxidation under oxidative stress**, and it is up-regulated by increasing concentrations of exogenous H_2O_2 at 37 °C (34).

Another form of iron use is the one described for *L. enriettii*, in a **multidrug resistance gene (*MDR*)**. This gene *LeMDR1* (*L. enriettii* multi-drug resistance gene 1), mediates resistance to **vinblastine**, demonstrated by association studies, transfection and *knock out* (KO) assays. The *LeMDR1* gene **encoded protein** was proposed to be **part of the drug resistance mechanism, by sequestering the drugs instead of actively secreting them outside the cell.**

When the Vint3 and V160 cell lines, that over-express the *LeMDR1* gene, were compared with wild type and double KO mutants for this gene (*LeMDR1-/-*), it was shown that the number of gene copies was associated with: 1) **a high level of intracellular iron**, 2) **a high sensitivity to iron-depending antibiotics such as streptonigrin** and 3) **an increase in the enzymatic activity of an iron-sulfur mitochondrial enzyme, aconitase**. These results suggest that the normal function of *LeMDR1* gene is related to homeostasis of mitochondrial iron. In these assays, using target specific drugs, such as vinblastine that targets the cytosol or **rhodamine 123** and **pentamidine** with specificity for mitochondria, it was possible to show that the resistance level of V160 cells to vinblastine increased with iron, whereas resistance to rhodamine 123 and pentamidine increased with iron depletion and *vice-versa* [35].

***Leishmania*, which is incapable of synthesizing heme, can endocytose Hemoglobin (Hb), using a specific receptor** that has been identified in *L. donovani* promastigotes [32]. Particularly in *L. donovani*, **Rab7 protein** present both in early and late endosomes, is involved in **Hb capture.** Over-expression of *Leishmania* Rab7 induces the transport of Hb to lysosomes where it is rapidly degraded, whereas in Rab7 mutants, this transport and degradation of Hb is diminished, with cells having 50% less growth when

compared with wild type cells. Exogenous **hemin** addition to Rab7 mutants recovers their optimal growth, suggesting that intracellular generated heme group from Hb is required for optimal parasite growth [36].

6. Conclusion

Currently, there is no information in this parasite concerning the existence of chelating or iron competing proteins or proteins that present protease type activity against the iron carrier or storage proteins from the host. Therefore, the parasite obtains iron after it has been released of their proteins by the host itself, making use of the own host machinery. The first contact of *Leishmania*, in its promastigote form, with iron is the capture of Hb by endocytosis mediated by Rab7 at the moment of infection in the lesion site caused by the vector, which then is degraded to use the heme group for the parasites' survival.

However, the main proportion of iron is captured intracellularly when the parasite has been established in the PVs. In this organelle with phagolysosome characteristics, the parasites maintain a pH around 5.5 also promoting a further fusion event between early endosomes and the PV, which provides the parasite with nutrients for their survival including Tf-bound iron. When the parasite has already acquired iron, it is distributed towards different compartments for further use (Figure 4). It is known that Fe is essential for survival and growth of the parasite.

Therefore, it is possible to conclude that by itself, iron and iron transporters may represent strategic drug targets for the design of therapeutic drugs for the control and eradication of infections produced by these intracellular parasites from the *Leishmania* genus.

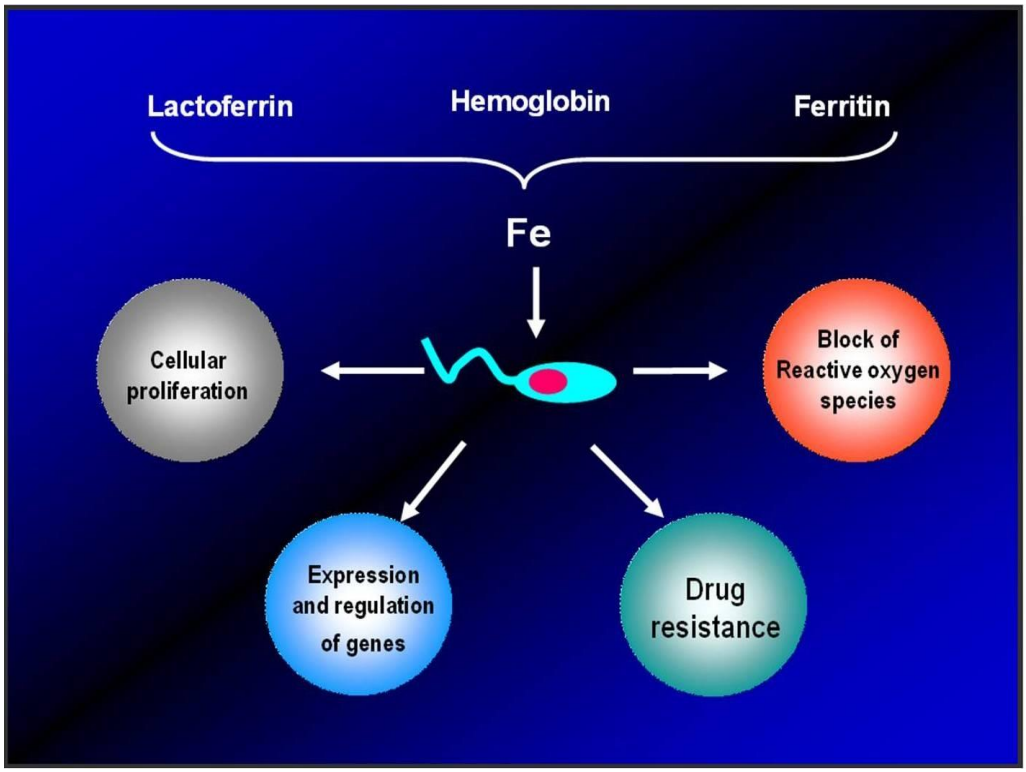

Figure 4. ROLE OF IRON IN *Leishmania*.

Iron acquisition from iron binding proteins. This metal is used for diverse cellular functions that allow the promastigotes of *Leishmania* to stay alive since the first contact with the host; later in its amastigote form, iron allows the parasite to modulate the immune response and persist in the host infected cells by its regulation of the multidrug resistance. Gene expression is a key element that allows the parasite to mediate the cellular functions important for its survival during the host-parasite interphase.

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Chapter 19

Helminths and iron

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Abstract

Introduction

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Abstract

Intestinal parasitosis is a very frequent infection in children of developing countries. It is estimated that over 3,500 million people worldwide are parasitized and 450 million suffer from some related disease.

The parasites that cause more deaths in the world are *Schistosoma sp*., *Ancylostoma duodenale*, *Necator americanus*, *Trichuris trichiura* and *Ascaris lumbricoides*. These organisms generally fed on the blood of their hosts; therefore, they can cause severe anemia in children, pregnant women, and immunosuppressed people.

Both free-living and parasitic helminths require Heme to live. Free-living helminths can synthesize Heme but parasitic helminths lack this capacity, so they must obtain Heme from the host.

In this chapter we review some of the mechanisms by which pathogenic helminths can obtain iron from their hosts.

Keywords: cestodes, cytochrome oxidase, fumarate reductase, iron uptake, nematodes, succinate dehydrogenase, trematodes.

Introduction

Intestinal parasites are the most common infectious agents in human beings. They are disseminated widely around the world; nevertheless, tropical and subtropical countries gather the climatologic characteristics that favor the prevalence of the majority of these organisms [1].

Intestinal parasitosis is a frequent infection in children of developing countries, where often this disease is **a poverty indicator**. As it is known, the bad sanitary conditions, low socioeconomic level, low water availability, deficient elimination of excreta, and the climate, are associated to a high prevalence of parasitism [2].

It is estimated that around 3,500 million of the world population is parasitized and approximately 450 million of them suffer a related disease. As mentioned earlier, a large proportion corresponds to children. According to recent data proportioned by the WHO, 75,000 annual deaths are caused by infections with nematodes such as ***Schistosoma spp., Ancylostoma duodenale, Necator americanus,*** **and** ***Trichuris trichiura,*** and other 60,000 deaths are related to ***Ascaris lumbricoides*** [3,4].

Helminths are classified within the Aschelminthes and Platyhelminthes phyla. The **Nematode** class (roundworms) belongs to the first phylum and the **Cestoda** (round and segmented worms) and **Trematoda** (flatworms) classes belong to the later [5].

Helminths have complex life cycles. These extracellular organisms can live in the human intestine, blood vessels, lymphatic organs, and other organs or tissues. **Frequently, helminths migrate through tissues until reaching the definitive organ in which they settle.** Their purpose is not to kill the host; instead they can survive many years in their hosts because they have developed strategies to evade the immune responses [6].

According to their life stage, helminths show different morphology. For example, the **cercarias** of *Schistosoma sp*. lose their branched tail needed in an aquatic environment once entering the host. When the parasites enter to the host, they need an **adaptation in their metabolism** passing from an aerobic environment to a facultative anaerobic environment. In the migration through the tissues, they are exposed to stressful conditions due to changes of temperature, pH, oxidative products, or free radicals. In all these conditions, parasites must adapt themselves; therefore, they have a **continuous activation of genes** that leads to the expression of molecules that help them to this adaptation [7].

Iron is an essential element for the body functioning, since it is a basic component of numerous molecules whose functions include the oxygen transport, enzymatic reactions (that bind the metal in form of free ions, heme groups, and iron-sulfur groups), immunity, and regulation of corporal temperature. Moreover,

iron plays an important role in the maturation of the nervous system, in the development of intellectual abilities, and in the conduct patterns [8].

The Heme group of Hemoglobin (Hb) and the Ferritin are the most abundant iron sources in mammalian hosts. The mechanisms for Heme uptake have been described in both Gram-positive and Gram-negative bacteria; nevertheless, the Heme transportation systems in eukaryotes are less known.

It is also known that **iron regulation in the mammalian host displays an obstacle for invading pathogens, which require this essential element.** In the host, iron is bound to proteins or inorganic **chelates** as a strategy of the **innate immunity**. Thus, successful parasites must **scavenge iron** from the **host resources, which** is a decisive factor in the infection. Most of the circulating iron in blood is in form of Heme group (bound to Hb within erythrocytes), or is reversibly bound to iron carriers such as **Transferrin (Tf)** [9,10,11].

Parasitic and free-living helminths require Heme for survival. Free-living helminths can synthesize this type of chemical group, but the parasitic organisms lack the capacity to synthesize Heme, thus it must be obtained from the host. Heme transporters in these organisms have not been identified [12].

Protozoa and helminths are iron-dependent; nevertheless, some groups of parasites have lost their ability to acquire iron as they have been adapted to a parasitic life [13].

The first universal precursor for the synthesis of Heme is the δ-Aminolevulinic acid. The synthesis of Heme ends when **Ferrochelatase** catalyzes the ferrous iron insertion into the ring of **protoporphyrin IX** to form **protoHeme**. ProtoHeme is incorporated into numerous **hemoproteins** or is modified for the synthesis of other types of Heme groups found in **Cytochromes c** and **Terminal oxidases**. A genome analysis of several helminths shows that parasitic worms lack orthologous genes that codify for the Heme group biosynthesis [14].

On the other hand, **anemia** is a frequent blood disorder that occurs when the number of red blood cells (RBCs) is lower than the normal, or when there is a low concentration of Hb. Frequently, anemia is a disease symptom more than a disease itself, and it has been correlated with helminths infection that causes mainly the **Ferropenic anemia**.

The most frequent types of anemia are the **Hypochromic Microcytic Anemia** (the most frequent is by iron deficiency), **Normochromic Macrocytic Anemia** (where megaloblastic anemia is also related to a deficit of folic acid or B12 vitamin), and the **Normochromic Normocytic Anemia** (in which hemoglobinopathy, spherocytosis, and Glucose-6-phosphate dehydrogenase deficiency are found) [15].

Iron deficiency is a public health problem. At the global level, about 2,150 million people suffer from deficiency of this element and of these more than 50% suffer anemia. In developing countries, the infant

undernourishment constitutes one of the main causes of mortality. Malnutrition and iron deficiency with or without anemia are common and usually associated phenomena [3,16]; both undernourishment and iron deficiency are disorders whose cause is similar, being the most frequent: food inadequate ingestion, high physiological demands, deficient intestinal absorption, and chronic blood loss. The chronic loss of blood may be due to the presence of inadvertent hemorrhages of the digestive tract, which can occur in children with severe infections caused by certain intestinal parasites such as helminths. Several studies have demonstrated that the presence of intestinal parasites is correlated to the undernourishment and Ferropenic anemia in children [3,17,18].

Furthermore, **anemia by iron deficiency has been associated with negative effects on the cognitive development, school performance, child growth, resistance to infections, low birth weight, and maternal and infant morbidity-mortality** [16,19,20,21]. In developing countries, inadequate consumption of good iron sources is indicated as the main reason for the high prevalence of anemia, but another important factor to be considered is the infection by helminths in children [18].

1. Nematodes (round worms with thin ends)

There are four main species of nematodes that infect humans and are mainly transmitted by the parasite´s eggs that are on the soil: ***Ascaris lumbricoides* (roundworm), *Trichuris trichiura* (roundworm, whip-shaped), and *Ancylostoma duodenale* and *Necator americanus* (hookworms).** After infective larvae are ingested with the eggs (*A. lumbricoides* and *T. trichiura*) or penetrate the skin, they moult and develop into adult in the large intestine (*T. trichiura*) or in the small intestine after they have migrated through the respiratory tract (*A. lumbricoides*).

This type of parasites can cause anemia in humans, because they induce a very important gastrointestinal loss of blood, as do *Ancylostoma duodenale* and *Necator americanus*, since worms take directly the Heme group from the host blood causing Ferropenic anemia. In this case, **the anemia is caused by active feeding of blood from damaged blood capillaries in the intestinal mucosa, resulting in significant gastrointestinal hemorrhages, loss of serum proteins, and intestinal inflammation** [22,23].

Concerning *A. lumbricoides* and *T. trichiura*, the mechanisms for Heme uptake are not yet known. Unlike the hookworms, **these do not produce intestinal bleedings but acquire Heme from their adhesion to host muscles** [2]. It has been described that in muscle, the complex II of mitochondria of adult stage of *A. suum* (worm that infects pigs) works as a terminal oxidase (**Fumarate reductase**) and donates electrons to the fumarate, which is the terminal acceptor under anaerobic conditions. In contrast, complex II of the larval stage L2 shows lower activity of the Fumarate reductase than the enzyme of the adult stage and works like a **Succinate dehydrogenase** in the aerobic life [24,25].

On the other hand**, Cytochrome oxidase** has been detected in larvae of *A. suum*. Thus the parasite adapts to changes in its environment and physiological conditions of life cycle by modulating its energy metabolism including terminals of oxidase system. However, it is not clear the enzyme participation in electron transfer within the mitochondrial complex II [26].

Ancylostoma duodenale and *Necator americanus* have an economic and social importance in a large number of countries. They cause the **neglected tropical diseases** (NTD) also called **ancylostomiasis and necatoriasis,** respectively. These parasites cause iron deficiency anemia, which can cause physical and mental retardation, children death, and abortions [27].

Stephenson and colleagues have demonstrated the cumulative effect of this type of hookworms and the iron deficiency in children and women in childbearing age who can have negative effects in their growth and mental development [28]. For that reason, some strategies have begun to develop to control the infection caused by these worms as well as to fight the undernourishment in children, where regularly it provides a combined therapy of anti-helminths and iron supplements [23]. It is important to mention that each of these parasites can cause a daily hematic loss between 0.03 and 0.26 ml per worm.

On the other hand, there are studies over *Ancylostoma caninum* describing that the adult form attaches to the intestinal mucosa of its host, the worm lacerates blood vessels surface and sucks the blood towards its buccal capsule. It has been clear for near a century that the worms of *Ancylostoma spp*. produce powerful **thrombosis inhibitors**; although it was recently known the exact mechanisms by which these **hematophage nematodes** prevent the formation of clots. Currently, two **anticoagulants** related to inhibitors of serine proteases have been isolated from *A. caninum*. These 9 kDa proteins called **anti-coagulating peptide 5 and C2 peptide are powerful and specific inhibitors of the coagulation factor Xa and the complex tissue factor VIIa, respectively** [29].

2. Cestodes (round and segmented worms)

The species that mainly affect human beings are *Taenia saginata* (intermediary in cattle) and *Taenia solium* (intermediary in pigs, **cysticercus**).

T. solium persists several years in the human central nervous system. A study has revealed the identification of different protease activities including **Metalloproteinases**, **Cysteine proteases** and **Aspartic proteases** [30]. These enzymes help the parasites to survive in the host because they have the ability to digest Hemoglobin and IgG (they preferably degrade the heavy chain).

3. Trematodes (flat worms)

In the Trematoda class are included *Fasciola hepatica* and *Schistosoma spp*. Recently, a **Thioredoxin peroxidase** (TPX) was isolated from a *F. hepatica* cDNA library [31]; nevertheless, it is unknown whether this antioxidant protein actually protects sensitive enzymes against metal-catalyzed oxidation systems (MCO). However, Salazar-Calderón and colleagues found that this TPX is able to protect several enzymes from inactivity caused by MCO systems.

In contrast to all known TPX enzymes, the *F. hepatica* enzyme is able to protect to **Enolase** and the **Glutamyl synthetase** from inactivation by the $Fe^{3+/}O_2$/ascorbic system, suggesting functional differences on many TPX, which are thiol-specific enzymes [32]. In addition, it is also known that *F. hepatica* presents one cysteine protease with **hemoglobinolytic** activity and it is located in the granules of the intestinal epithelial cells. This molecule is secreted into the intestine lumen and is involved in extracellular degradation of the host Hb [33].

The adult worms of *Schistosoma mansoni* have a hemoglobinolytic activity essentially due to the expression of a **Cathepsin type L protease** [34]. A second study identified a **Cathepsin type B protease** denominated Sj31 and a **Hemoglutinase** of an adult stage of *S. japonicum*. In male worms, the location of Sj31 in the intestinal wall and lumen has been demonstrated by immunofluorescence. Cathepsin B has great similarities with the Sm31 Cysteine protease identified in *S. mansoni* as well as in *S. haematobium*.

There is solid evidence that iron is used by *Schistosoma* parasites for their development and reproduction. *Schistosoma* species take iron in the **gastrodermal lumen** where they store a great amount of **Heme and Hematin**. A great amount of iron is also stored within **isoforms** of the highly conserved iron storage protein, called **Ferritin**. One isoform, Fer-2 is typical of somatic tissues; the other, Fer-1 appears as an abundant component of platelets of vitelline cells, egg wall precursors, and possible yolk cells. The female *Schistosoma* parasites express 15 times more Fer-1 than males do but both genders express the same amounts of Fer-2 [34].

The roles for abundant iron storage associated to eggs include the early **embryogenesis** and stabilization of protein crossed in the formation of egg cortex. **Transferrin (Tf) and the iron bound to non-Transferrin molecules (NTBI) stimulate the growth of *Schistosoma spp. in vitro*.** The stimulatory effect of NTBI can be reverted in the presence of the iron chelator **Deferoxamine**. However, *Schistosoma spp*. can acquire iron through mechanisms dependent and independent on Tf. The attached Tf by the **tegument** is neither saturable nor specific; therefore, it excludes the action from specific Tf receptors.

In contrast to mammalian cells and trypanosomes, *S. mansoni* expresses two isoforms of a **Divalent Metal Transporter** (DMT-1) with similar sequence to the protein taking ferrous iron in mammals. Remarkably, the DMT1 of *Schistosoma spp*. has been located in the tegument and not in gastrodermis. This location pattern, complemented with *in vitro* studies realized by Clemens and Basch [37], suggests that iron

taking occurs on the cell surface and probably through iron transporters such as Tf, which is abundant in the host serum. Furthermore, since DMT transports Fe^{2+}, it is likely that one **Ferric reductase** is required for taking iron but to date none has been identified [38].

Besides its high sequence identity with mammalian proteins, neither Ferritins nor DMT1 sequence of *Schistosoma spp*. has the associated region with post-transcriptional iron regulation found in the homologous sequence of mammals [35,36,37].

Rao and collaborators [14] suggest that helminths break the Heme from sources such as diet or environment. In the case of *Schistosoma spp*., as mentioned early, the gastrodermal lumen represents the main source of Heme, much of which is sequestered in the **Hematin** [14]. It is important to mention that the sequences of enzymes known for the biosynthesis of Heme such as **δ-Aminolevulinic acid dehydratase (ALAD)** and the **Porphobilinogen deaminase (PBGD)** have been reported in the tag data base (EST) for *Schistosoma spp*., suggesting that helminths have the ability to synthesize Heme. If the source of Heme for hematophagous helminths is *de novo* or salvage is an intriguing question without solving. Also, it remains to be determined if the helminths can store iron from Heme through the **Heme-oxygenase** [14].

Renewed interest in how parasites acquire Heme leads to the question of whether *Schistosoma spp*. can use the host Heme for Hemoproteins synthesis or as an iron source. The gastrodermis is enriched with Heme under the hemoglobinolytic routes. It has been thought that *Schistosoma spp*., as well as other parasites do not have the capacity to digest Heme emptying from the stomach as Hematin by regurgitation. There is no evidence *in silica* that the Heme is metabolized to release iron, due to there is no tag data base (EST) representing Heme-oxygenases.

Nevertheless, Glanfield and colleagues [38] have described heme-oxygenase activity in *S. japonicum.* This requires confirmation given the data accumulation on the iron-taking dependent on the Heme group in metazoans and the reasonable hypothesis that the excess of Heme in the stomach of the *Schistosoma spp*. can act like an iron source. Therefore, it has been reached the conclusion that an investigation over the mechanisms to use Heme is justified and necessary [38].

Finally, we conclude that to date the mechanism by which helminths acquire iron from their hosts is poorly known. Further studies are needed to know if there are iron carriers, enzymatic mechanisms to obtain iron from Heme, or other mechanisms. The parasitism by helminths needs to be more studied to understand the impact they have in life quality, children development, and pregnant women risk in developing countries, mainly in tropical areas, while deworming programs and iron nutritional supplements are provided.

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Chapter 20

Iron, parasites, and animal models

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Abstract

Acquisition of iron plays an important role in host-parasite interactions in many diseases. In order to understand the mechanisms for iron acquisition, several authors have established animal models some of which are explained briefly in this chapter.

The siderophores, the uptake of heme groups, and some iron transport proteins have been studied to describe how the parasites get iron from the host.

Keywords: mouse model for malaria, mouse and *Ascaris suum*, transferrin and *Trypanosoma brucei*.

1. Introduction

Wessler suggested in 1976 that the definition of an animal model for the study of human diseases is the following: **"A living organism with an inherited, acquired naturally, or induced pathological process, by which one or several aspects resembling the same phenomenon in human beings ".**

This phrase adequately describes the parasitic infection in animal models. The choice of the animal model depends on **the type of disease** (infection) to be studied and **the hypothesis to be tested** in the model. In parasitic infections, the choice also depends on the appropriate parasite-host combination that currently exists or can be established experimentally. An appropriate model should mimic **(a) the host, (b) the parasite, and (c) the host-parasite system or the form in which the parasite and host interact** [1].

Studies in animal models have obvious advantages over direct studies in humans: The animal care is relatively easy and less expensive by case and equivalent data (except for studies in primates). Moreover, experimentation in animals is easier to handle and ethically less problematic than the work with humans, mainly when it is carried out on animals that will be consumed by humans [1].

The choice of a suitable host for an appropriate infection model is well done if we examine the **immunological, physiological, anatomical, and metabolic similarities** over the animal models against the natural host since all these characteristics can influence the experimental results. It is desirable that all these features be as similar as possible to choose a specific animal model.

On the other hand, finding equivalent parasites of animals from the four main intestinal nematodes infecting humans is not difficult: **helminths of the genera *Trichuris*, *Ascaris*, *Strongyloides*, and *Ancylostoma* occur in closely related species in animals and humans.**

The most important point is that animal models should be chosen as similar as possible from natural parasite-host systems. Naturally occurring ***Trichuris muris* in mice, *Ascaris suum* in pigs, and *Ancylostoma caninum* in dogs** are well described infections and they are nearly related to parasitic species that infect humans; thus, they can serve as good models to study the corresponding human infections. Other natural models like *Trichuris suis* and *Strongyloides ransoni* in young pigs also have been suggested as a study for a parasite-host relationship [2].

As already mentioned, rodents are easier to handle and cheaper to use them in parasitism models. There are examples that ***Trichuris muris* and *Trichinella spiralis* can reproduce in mice**; nevertheless, the problem is that these models cannot be used for great size parasites like *Ascaris* and *Toxocara* due to size limitations. In contrast, larval stages of these parasites can be studied in mouse models.

For instance, ***Ascaris suum* larvae can be satisfactorily established in guinea pigs and in mice, and also *Trichuris canis* can be established in mice** [1]. Thus, the larval migration in rodents can be used in infectivity studies that do not require permeability. Infections with ***Schistosoma mansoni*, *S. japonicum*, and *Plasmodium spp*. (malaria) have been studied in a small number of chimpanzees** while the **immune response to infections of *S. mansoni* and *S. haematobium* has been systematically studied in baboons** [3].

I**ron acquisition** plays an important role in the parasite-host interactions during many microbial infections. To take care of iron, vertebrates have developed an elaborated defense system which must be overcome by the pathogen invaders. To meet this aim, pathogens have evolved several strategies that include the secretion and binding of **Siderophores**, the uptake of host **Heme groups**, **the binding of host Iron transporting proteins**, or **accessing to the host Intracellular iron reserves** [4,5]. The efficiency of the mechanisms used by pathogens to obtain iron retained by the host may vary between strains of a species and represents an important virulence factor in bacteria and other parasites [5].

2. Iron-binding proteins and sites of iron storage in the host

2.1 Conalbumin (Ovotransferrin)

This 77 kDa macromolecule consists of a polypeptide chain of approximately 680 amino acids besides an oligosaccharide fraction of four mannose and six glucosamine residues. Two iron-binding sites are present in this polypeptide. Three tyrosyl (or tryptophanyl) and two residues of histidinyl besides an ion carbonate serves as ligands for each metal binding site. Although all tested transition metals can be attached to the protein, their avidity for iron is greater than for other ions.

2.2 Lactoferrin

Like Conalbumin, Lactoferrin is a glycoprotein. This consists of a single polypeptide chain of approximately 680 amino acids with two identical carbohydrate chains bound together. An important property of Lactoferrin is its ability to bind iron when the pH drops below 4. Since Lactoferrin is an iron chelator more potent than Transferrin in the plasma, it maybe conceivable that Lactoferrin participates in the iron transference from plasma to milk in nursing women, so newborns excrete up to ten times more iron than one month old babies.

2.3 Transferrin

This molecule contains approximately 680 amino acids and a carbohydrate chain. The human Transferrin consists of two identical ramified chains bound together to asparagine residues; each contains two sialic acids, two galactoses, three mannoses, and 4 N-acetyl glucosamine residues. The groups that bind iron in the polypeptide chain consist of three tyrosyl and two histidyl residues besides ion carbonate; thus each molecule has two iron-binding sites as well as Conalbumin-binding sites.

Unlike Lactoferrin, Transferrin requires pH values above 6.5 for a maximum iron-binding activity. In humans, the normal value of iron saturation varies with age: the higher value is 69% and the lower value is 22%. The normal value also varies between species; for example, the average values in cattle, mice, rabbits, and guinea pig have been reported of 39%, 60%, 64%, and 84% respectively. Moreover, the observed values in turtles, frogs, rats and chickens were of 24, 24, 34 and 47%, respectively [4].

3. Protozoa and helminths models

3.1 *Giardia lamblia* and *Entamoeba histolytica*

Cheeramakara and colleagues found that, in a model of *Giardia lamblia* infection in rats, anemia was caused in the animals due to a poor absorption of nutrients from the diet since the protozoan causes a severe damage in the small intestine affecting the absorptive capacity. This infection is very similar to those affecting human beings [7].

Entamoeba histolytica multiplies in the large intestine, causing dysentery. This amoeba sometimes travels to the liver and other organs. In the liver, it causes hepatic abscesses that can be fatal. A different animal model has been developed for studying amoebiasis in the large intestine and the liver. Human intestinal amoebiasis has been reproduced by intracecal infection of the sensitive C3H/HeJ mouse strain. Hepatic abscesses can be reproduced in the Sirian golden hamster. In both models the infection and immunological implications as well as trials with anti-amoebic treatments have been assayed [8, 9]

3.2 *Tritrichomonas foetus*

This parasite is the etiologic agent of bovine trichomoniasis, a contagious venereal disease in cattle. This protozoan has hydrogenosomes which have relatively high requirements for iron (50-100 μM) due to the importance of proteins that contain iron in its metabolism [10].

Kulda and colleagues found that *T. foetus* can accumulate iron from Lactoferrin, Transferrin, and low molecular weight iron-complexes of the host. They also demonstrated that the host mortality increases when mice are infected with *T. foetus* (median and high virulence strains) and then fed with an iron-rich diet. When the high virulence strain is inoculated without the iron supplement, a similar phenomenon take place [11].

3.3 *Trypanosoma brucei* and Transferrin

Most parasites cannot use the natural iron reserves of the host like Transferrin. However, trypanosomes are able to use it as an iron source. *Trypanosoma brucei* is a parasitic protozoan that lives extracellularly in the blood and tissues of a wide range of mammals. The *T. brucei* bloodstream forms find the required iron via the Transferrin receptor-mediated endocytosis into the host. The Transferrin receptor of *T. brucei* (TbTfR) is a heterodimeric protein complex bound to a glycosylated phosphatidylinositol (GPI) that binds a Transferrin molecule [12].

Parasitic infections can lead to iron deficiency anemia which is characterized by a reduction in the iron saturation of Transferrin from a normal value of 30% to 15%. Previously, it has been reported that in a rabbit infection with *T. brucei*, the plasma iron (which mainly represents the amount of iron bound to Transferrin) is decreased approximately 52% compared to pre-infection levels [13].

3.4 *Trypanosoma cruzi*

Lalonde and Holbein showed in 1984 that the reduction in the iron storage by the iron chelator Deferoxamine B and an iron-deficient diet provide complete protection to mice infected with *T. cruzi* [14]. Later, Deharo and colleagues demonstrated that some iron chelators like Aminothiol multidentate chelators also inhibit *Trypanosoma* parasites [15].

4. Iron chelators in malaria and in mouse models

Plasmodium falciparium has shown an increase in resistance to antimalarial drugs; thus, new compounds are being used to reduce this resistance, including iron chelators. However, these drugs display some disadvantages like their high hydrophobicity, length of stay in the host system, and the high toxicity to the host. At the moment, the only iron chelator drug used in humans is Deferoxamine (DFO).

Aminothiol multidentate chelators also inhibit malaria parasites. Nowadays, infection models with *Plasmodium spp*. are being used in mice in order to improve the transport and release of DFO along with

other drugs that act synergistically, such as the lipophilic salicylaldehyde isonicotinoyl hydrazone (SIH) that has demonstrated to inhibit the proliferation of *P. falciparum in vitro* [16].

It is important to mention that iron chelators can act in several ways:

a) Deprivation of the iron source for the parasite,

b) Iron dissociation from Hemoglobin which releases toxic iron molecules and therefore induces the synthesis of lethal reactive oxygen species, and

c) Direct inhibition of metalloenzymes and other enzymes [6].

4.1 Effects of iron supplementation on malaria infection

There is a very important paradox in malaria endemic regions; the iron deficient diet of the inhabitants facilitates the infection by *P. falciparium* which produces anemia that is regularly treated with iron supplement as recommended by the World Health Organization. Nevertheless, this treatment increases the amount of parasites in the host. Therefore, **it is necessary an initial treatment to deworm the host and later the iron supplementation in the diet.** To study such treatments, several study models have been developed, the most common is in young Wistar rats [17].

5. *Ascaris suum*

Mice infected with *A. suum* and fed with a low iron diet showed a low number of larvae in the lungs compared with mice that received normal or light iron diets [18].

6. Hookworms

The hookworm infection is one of the most prevalent parasitic diseases since it infects around 740 million people in tropical and subtropical regions of the world [19, 20]. Worldwide, *Necator americanus* is the predominant etiologic agent in humans infected by hookworms, whereas *Ancylostoma duodenale* has more occurrences in scattered focal environments. Other hookworms like *Ancylostoma ceylanicum*, *A. caninum*, and *A. braziliense* infect humans as a zoonosis, but they are not considered a very serious public health problem.

The higher prevalence of hookworms happens mainly in sub-Saharan Africa and East Asia. In all of these regions, there is a close relationship between hookworms and low socioeconomic status. The clinical symptoms of hookworm infection include Ferropenic anemia and protein-losing enteropathy that may lead a

mental and physical developmental delay [21]. Chemotherapy is effective at eliminating existing adult stage parasites; however, reinfection can occur rapidly after treatment [22]. This observation, along with the epidemiology of hookworm infection as well as the fact that the number or worms increases with age in endemic regions, suggests that hookworms can both evade or suppress the host immune response.

6.1 Animal models for hookworms

In recent decades, there have been many attempts to create animal models for hookworm infection. These experimental models are needed to investigate new vaccines and anthelmintic therapies. On the other hand, hookworm infections have been tried in species such as rabbits [23], chickens [24], or chimpanzees [25]. Mice have also been defied with different species of *Ancylostoma spp*. and *N. americanus*; however, they are not permissive for the development of adult stage so have no blood loss. For this reason, many researchers consider that the mouse model for hookworm is not physiological [26] and they can be only useful to evaluate numerous chemotherapeutic agents and candidate vaccines [27].

Perhaps the most appropriate models to study the hookworm infection are natural or permissive models such as dogs and hamsters. Dogs can be infected experimentally with *Ancylostoma celanicum* and *A. caninum* while hamsters can be infected with *A. celanicum* and *N. americanus* [28].

7. Schistosomiasis and anemia

The mechanisms by which infections lead to anemia have been debated for decades. The four proposed mechanisms are: 1) Iron deficiency due to extra-corporeal loss, 2) splenic sequestration, 3) autoimmune hemolysis and 4) anemia of inflammation.

1) Extra-corporeal loss of iron. In 1982, Mahmoud and colleagues demonstrated that infections with adult worms of *Schistosoma mansoni* are responsible for anemia. It has been demonstrated that loss of blood is due to the presence of the parasite and not by the presence or liberation of eggs given that previously was thought they caused the rupture of intestinal walls and hemorrhages, and consequently anemia [29].

2) Splenic sequestration. In a mouse model infected with *S. mansoni*, it was demonstrated that the spleen increases in weight and tends to retain erythrocytes in this inflamed organ.

3) Autoimmune hemolysis. It has been found that *S. mansoni* breaks erythrocytes causing an immune response against the erythrocytes residues and producing an autoimmune response that can increase the loss of these cells (anemia). This mechanism has been observed in mice without spleen given that splenomegaly cannot explain the loss of erythrocytes.

4) Anemia of inflammation. In a rabbit model infected with *S. japonicum* were found high levels of proinflammatory cytokines such as IL-1, IL6, C-reactive protein, and TNFα. TNFα production causes a low production of Erythropoietin; therefore, there is a growth inhibition of stem cells from bone marrow. In turn, IL-6 causes an overexpression of Hepcidin which diminishes iron absorption in the stomach, alters the iron metabolism, and causes sequestration of the molecule. Iron is kept within cells in storage forms as Ferritin in the reticule-endothelial system decreasing its bioavailability [30, 31].

8. Conclusions

Animal models to study iron uptake in the parasite-host interaction should take into account several factors. While in humans exist nutrimental deficiencies (low amount of iron), in animals this process does not occur naturally. In animal models, a low iron diet does not facilitate a successful parasite infection. The internal environment is less favorable for parasites to settle because essential elements like iron are not available for them. A well-established animal model must have well fed animals for a successful parasite infection. Once the infection is established, the experiments can be realized focused on the study of malnutrition (iron metabolism), immunological aspects, and therapeutic treatments, among others.

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D. Outlook

Undoubtedly, the struggle for iron between diferent organisms in order to survive, is a fascinating subject. The phenomena by which increasing the concentration of iron in the host led to an increase of the infection by invading pathogens was described approximately 40 years ago. Since then, and very rapidly in the last ten years, this field has been growing and now exceeds the number of items in much the ability to read and assimilate.

We know, partially, some of the participants in the struggle for iron, and the mechanisms used by them to try to win the battle. Much remains to be learned about the molecules of different cells that act as receptor proteins that contain iron, as well as the signal transduction pathways required to start up the cellular mechanisms of reception and assimilation of iron, both in pathogens and in host cells.

In the future, it will be necessary to extend the knowledge about the cellular processes of iron acquisition by pathogenic organisms, as it will take to learn how to avoid requiring iron within the host. The design of vaccines directed against microbial siderophore receptor proteins or iron is a process essential to continue, as one of the strategies to counteract the harmful effects of these microorganisms.

Moreover, natural or synthetic substances capable of chelating iron and keep it unavailable to pathogens, such as siderophores, transferrin, and lactoferrin have already been used in some cases with a certain level of success. Therefore, research on these products continues, in order to improve their effect and to avoid side effects and adverse reactions, and the development of resistance mechanisms by pathogens.

Finally, we invite research groups to join scientists already contributing to expand knowledge about the importance of iron for living beings; surely the issue and the challenges involved will be of much interest.