Chapter 11

Hepcidin: SNP-Like Polymorphisms Present in Iron Metabolism and Clinical Complications of Iron Accumulation and Deficiency

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Abstract

The metabolism of iron is regulated by the peptide hormone hepcidin. Genetic alterations in the proteins involved in the signalling pathway and hepcidin transcription cause damage to the organism. Mutations and polymorphisms in the hepcidin antimicrobial peptide(HAMP), HFE, HJV, ferroportin and matriptase-2 genes influence serum hepcidin concentration. Genetic deficiency of hepcidin increases iron overload in tissues, leading to haemochromatosis. However, genetics changes in the TMPRSS6 gene promote an increase in serum hepcidin, with the development of severe anaemia and resistance to iron treatment, as observed in IRIDA. Making the flow and efflux of extracellular and intracellular iron is impossible. To date, no drug that works by inhibiting or enhancing hepcidin transcription is available, largely because of the cytotoxicity described *in vitro* models. The proposed therapeutic targets are still in the early stages of clinical trials, some are good candidates, such as heparin derivatives and mini-hepcidins.

Keywords: anaemia, ferroportin gene, HAMP gene, haemojuvelin gene, haemochromatosis hepcidin, HFE gene, IRIDA, iron homeostasis, polymorphism, TMPRSS6 gene, transferrin gene

1. Introduction

Disorders related to iron metabolism involve genetic alterations between hepcidin and the modulation pathway of the HAMP gene causing damage to the organism, and iron overload

or deficiency may occur [1–3]. Intracellular iron excess, hyperferremia, contributes to the formation of reactive oxygen species (ROS), damaging cell membranes and tissues, especially cardiac, endocrine and hepatic tissue, which are the clinical alterations observed in hereditary haemochromatosis [4]. However, iron deficiency due to genetic alterations, with an increased synthesis of hepcidin, reduces the synthesis of haemoglobin, limiting the formation of erythroid precursors and reduction of iron stores, with the development of anaemia [5, 6]. Hepcidin inhibits food absorption of iron in the duodenum, by blocking the release of iron recycled by macrophages and controlling the movement of iron stores contained in hepatocytes [7]. Thus, systemic iron stores, high concentration of plasma transferrin, erythropoietic activity, inflammatory mediators and hypoxia modulate the synthesis of hepcidin [8]. Plasma iron content is the main factor for transcription of hepcidin mRNA into hepatic tissue. When serum iron decreases, there is also a decrease in hepcidin transcription in the liver [9, 10].

The *HAMP* gene encodes hepcidin [1]. Studies have shown that changes in this gene or in genes that act on iron metabolism, by regulating the expression of hepcidin, influence the pathogenesis of hereditary diseases. Juvenile hereditary haemochromatosis originates from a polymorphism present in the HAMP gene, where deficiency occurs in the synthesis of hepcidin, with accumulation of iron in tissues [11–13]. Other factors, not related to genetic alterations, influence the serum concentration of hepcidin, such as viral infections and bacterins, vitamins A, D, C and E, hormones, stress of the endoplasmic reticulum, among others [14].

2. Hepcidin

The hepcidin molecule ('hep' hepatic origin, 'cidin' antimicrobial activity) was described in the year 2000, being characterized as a new anti-microbial peptide acting in part on innate immunity, such as β-defensin. Initially called liver-expressed antimicrobial peptide 1 (LEAP-1), it was isolated from human blood ultrafiltrates and urine in their active form, as a cysteine-rich peptide synthesized in the liver [1, 2]. Adipocytes, macrophages, lymphocytes, neutrophils, pancreatic β-cells and renal cells also produce hepcidin [15–19].

Physiologically active hepcidin originates from a pre-prohepcidin, containing 84 amino acids, which after proteolytic cleavage gives rise to pro-hepcidin that is composed of 64 amino acids. Pro-hepcidin is biologically inactive and is cleaved subsequently by the enzyme furin in a specific NH2 region, to provide biologically active source hepcidin, composed of eight cysteine residues bound by four bisulfite bridges and containing 25 amino acids [1, 2, 18]. The active molecule of hepcidin degrades in the N-terminal portion by giving molecules of −20, −22 and −24 amino acids, being this region, essential for the connection to cause ferroportina to occur. The smaller peptides show a progressive loss of regulation of ferroportin, with accumulation of intracellular ferritin [20].

2.1. Iron metabolism

Senescent erythrocyte recycling provides about 90–95% of iron required for physiological functions and for erythropoiesis. Enterocytes present in the duodenum and the proximal portion of

the jejunum absorb about 1–2 mg of iron from the feed, which maintains the iron stores [21–23]. In the cytoplasmic membrane of enterocytes, apical ferric reductase enzymes are present, such as cytochrome b duodenal enzyme (dCytB). The dCytB promotes the reduction of ferric iron (Fe^{3+}) to its ferrous state (Fe^{2+}) and consequent mobilization of ferrous iron through the divalent metal transporter (DMT-1). The haem iron, coming from diet, is internalized by the haem carrier protein 1 (HCP-1) into the cells, where it is stored as ferritin [24, 25]. When serum iron concentration is low, mobilization of iron stores by the ferroportin to the extracellular medium occurs. The released iron is in its ferrous state and for binding to serum transferrin to occur, it must be oxidized to its ferric state, only the Fe^{3+} binds to transferrin. This oxidation reaction occurs through the action of oxidase enzymes: Hephaestin is present in enterocytes, ceruloplasmin, hepatocytes, plasma and zyklopen in the placenta. Then occurs the release of iron into the tissues [26–28].

Iron recycling by macrophages occurs through phagocytosis of senescent erythrocytes, haemoglobin phagocytosis and haem group of the intravascular haemolysis. When the haem group is internalized by the macrophage, it undergoes the action of the enzyme haem oxygenase (HO) and releases ferrous iron, which can be exported to the extra-cellular medium by ferroportin or stored as ferritin [29]. The highest concentration of iron in the body is stored as ferritin or haemosiderin in the liver, spleen, duodenum, bone marrow and other organs. The ferritin molecule is composed of 24 subunits with a spherical 'shell' shape, which accommodates about 4000 iron atoms. Ferroportin mediates the efflux of iron stores, being negatively regulated by hepcidin [30, 31].

2.2. Ferroportin

Ferroportin is a transmembrane protein composed of monomer dimers present in hepatocytes, enterocytes, macrophages, spleen and bone marrow, which regulates the amount of iron present in the extracellular and intracellular medium. By means of stimuli originated by elevated levels of serum iron, the hepcidin mRNA increase occurs [32]. Binding of the mature hepcidin peptide to ferroportin leads to binding of the tyrosine kinase Jak2 to each ferroportin monomer. After the binding of Jak2 to ferroportin, the autophosphorylation of Jak2 occurs, which then phosphorylates the ferroportin. Phosphorylated ferroportin binds to hepcidin, and both are internalized by cavity coated with clathrin within the cell. Once internalized, the phosphates are removed and ferroportin is ubiquitinated in amino acid lysine 253. The ubiquitination is necessary for the entry of ferroportin in multivesicular bodies, which by fusion with lysosomes provides the degradation of ferroportin. A mutation at residue 253 does not preclude the internalization of ferroportin, but decreases its degradation [33].

2.3. Gene regulation hepcidin

The HAMP gene located on chromosome 19q13 transcribes hepcidin mRNA. Factors modulate the transcription pathway of this gene, hypoxia, iron concentration, erythropoiesis, inflammation, anaemia, among others. These factors activate two major pathways that act directly on the gene. The first signalling pathway occurs through the induction of the pathway related to bone morphogenetic proteins (BMPs) and the second Janus kinase/signal transducer and activator of transcription (JAK/ STAT) signalling pathway is related to inflammation [2].

The protein (HFE) protein acts on the regulation of hepcidin transcription through its interaction with the transferrin receptor (TfR). HFE is displaced from TfR1 by high concentrations of the transferrin-iron complex [Tf-Fe^{3+}] to promote its interaction with the transferrin 2 receptor (TfR2). HFE and TfR2 bind the BMP co-receptor haemojuvelin (HJV) and activate HAMP gene transcription via the BMP/Son of the mothers against decapentaplegic(SMAD) [34]. This interaction induces phosphorylation of the activated BMP receptor, promoting an intracellular signalling cascade, by binding to a threonine/serine kinase type I and type II receptor complex [35]. Activated type II receptor activates type I receptor, which then transmits the signal to the SMAD regulatory receiver (R-SMAD), phosphorylating SMAD-1, SMAD-5 and SMAD-8. In this way, the formation of a transcription complex involving the SMAD-4 factor occurs. The activated complex moves to the nucleus in order to regulate gene transcription [36]. SMAD-4 and matriptase-2 protein act as suppressor of BMP/SMAD pathway activation. Matriptase-2 interacts with HJV and causes fragmentation [6, 37].

The regulation of the HAMP gene through the JAK/STAT pathway begins when specific ligands act on the JAKs generating a multimerization of their subunits. Erythropoietin and growth hormone associate with the receptor forming a homodimer, whereas inflammatory cytokines and interferons form a heterodimer [38]. Interleukin-6 (IL-6) binds to its receptor, which is formed by two subunits, one alpha subunit (IL-6-R) and another beta subunit (gp130). When IL-6 binds to IL-6-R, a dimerization of gp130 occurs which recruits the cytoplasmic JAK to phosphorylate the gp130 protein. After phosphorylation, STAT proteins (STAT-1 and STAT-3) bind to gp130 and autophosphorylate, then migrate to the nucleus, binding to gene-specific transcription sites, promoting increased transcription of hepcidin mRNA into hepatocytes [39, 40].

2.4. Genetic alterations with iron overload

Genetic alterations in the HFE gene have always been associated with haemochromatosis, a situation in which iron accumulation occurs in the body with the development of cirrhosis, hepatocarcinoma, diabetes and heart failure. The main hypothesis for the development of haemochromatosis, is the interaction of HFE protein and β2-microglobulin. These two proteins together form a complex with the transferrin-1 receptor (β_2M-HFE-TfR1), located in the crypt of the duodenal enterocytes, which regulates the iron absorption of the diet. The interaction between these three proteins affects the processes of iron utilisation, i.e. on the intensity of erythropoiesis and liver metabolism of iron. Genetic changes in the formation of this complex give rise to the clinical picture observed in haemochromatosis. However, after the description of hepcidin in iron metabolism, this initial theory was altered [41, 42].

New genes involved in iron metabolism have been reported, and now the hereditary haemochromatosis term encompasses two types, HFE haemochromatosis, which is characterized by presenting changes in the HFE gene, this being the most frequent in the caucasian population. In the second type, known as non-HFE haemochromatosis, other genes involved in metabolism are described, which by polymorphic genetic alterations or mutation generate the phenotype of iron overload. Non-HFE haemochromatosis, the HJV gene, the HAMP gene, the TFR gene and the ferroportin gene are present with variable clinical characteristics among the

diseases, but all have altered levels of hepcidin. Genetic alterations in ferritin and ceruloplasmin also promote accumulation of iron in the body with hyperferritinemia, but these diseases are of recessive genetic inheritance and rare in the population [40, 12]. The accumulation of iron in the body compromises the function of several organs. In some severe cases of the disease, where diagnosis and treatment started very late, hepatic failure and development of cirrhosis, cardiac problems, changes in metabolic hormones with the development of diabetes, pituitary gland involvement, gonads, joints, hyperpigmentation of the skin, abdominal pain, testicular atrophy [43].

The diagnosis of haemochromatosis involves clinical features, biochemical measurements and genetic testing. Liver biopsy is performed in specific cases [44]. Elevated levels of serum ferritin (FS) and transferrin-iron saturation (TS) and liver enzymes are markers that help to identify patients with haemochromatosis. Serum ferritin levels greater than 200 μg/L in women and 300 μg/L in men and transferrin saturation greater than 45% are indicative of haemochromatosis when associated with clinical symptoms. When serum ferritin and transferrin saturation are altered, it is necessary to perform a genetic mutation analysis of the HFE, HJV, HAMP, ferroportin and transferrin receptor genes to make the differential diagnosis between haemochromatosis 1, 2, 3 and 4 [45, 46].

2.4.1. HFE gene mutation

The most frequent change that leads to the origin of hereditary haemochromatosis type-1 is the polymorphism and mutations present in the HFE gene present in chromosome 6. The allelic frequency observed in the European population is 5–7% for homozygosis C282Y, which is found about 80–85% of patients presenting with the disease [46]. The single point mutation, 845G → A, in exon 4, in the HFE gene leads to the substitution of cysteine by tyrosine at position 282 in the haemochromatosis (HFE) protein, which is the most severe case of the disease. Another variant of the HFE gene is the allele 187C → G, in exon 2, where an aspartate is exchanged for a histidine in the HFE protein (H63D), this alteration is of minor clinical importance, being harmful when it appears in heterozygous C282Y/H63D. The S65C variant is less frequent. The third mutation of the HFE gene is the substitution of a serine for a cysteine at amino acid position 65, 193A → T, (S65C). The most serious clinical manifestations are heterozygosis, C282Y/S65C, etc. [47, 48].

The cysteine residue in the α3 domain of the HFE protein that is altered in variant A of the C282Y HFE gene is necessary, as it forms bisulphite bonds and interacts with β2-microgloblulin, increasing its expression on the cell surface. The H63D mutation is present in the α1 domain of the peptide bond of the HFE protein, causing it to decrease the affinity between HFE and the transferrin receptor [49, 50]. Another aggravating factor of this variant is that the activation of the BMP/SMAD pathway for the transcription of hepcidin in the HAMP gene does not occur, due to the inability of the HFE protein to bind to the transferrin receptor. Therefore, changes in this protein contributes to the systemic accumulation of iron, by preventing iron absorption from the diet and by inhibiting the transcription of hepcidin [51].

HFE gene polymorphisms are associated with other clinical manifestations [52]. The H63D genotype modified the association between lead and iron metabolism, so that increased lead

in the blood is associated with a higher iron content in the body or a lower concentration of transferrin, favouring lead poisoning [53]. The H63D polymorphism is attributed to a greater propensity to develop disorders in porphyrin metabolism, insulin resistance and diabetes development, as well as being associated with increased aggressiveness of hepatocarcinoma and pancreatic cancer [54, 55]. The H63D variant is associated with the development of neurodegenerative diseases, due to stress generated in the endoplasmic reticulum and iron accumulation in individuals with β-thalassemea [56, 57].

Iron overload in haemochromatosis type 1 is rarely observed in children. In a study with 42 boys and 41 girls, aged approximately 12 years, both diagnosed with the H63D HFE heretozygen gene, the measurement biochemical parameters for iron, ferritin, transferrin saturation and total iron binding capacity. All values were statistically higher in the H63D HFE individuals, when compared to the control group. H63D-HFE boys presented higher iron content than the mean concentration found in girls. This fact can be explained by the loss of blood in menstruation [58].

Iron accumulation and oxidative stress have been associated with the development of neurodegenerative diseases. Studies describe that the C282Y and H63D HFE polymorphisms contribute to the pathogenesis of Parkinson's disease and Alzheimer's disease [59]. However, two meta-analysis studies indicate that these polymorphisms do not contribute to the development of these diseases [60, 61]. The C282Y genotype is related to propensity for the development of hepatocarcinoma, amyotrophic lateral sclerosis, non-fatty liver disease and venous ulceration, even in patients who do not show signs and symptoms of haemochromatosis [62–65]. Lifestyle, epigenetic factors, diet, alcoholism contribute to the development of iron overload in patients presenting the HFE gene C282Y, H63D and S65C polymorphisms. The diagnosis and early monitoring of iron overload indicators is necessary to reduce the damage caused by iron to the body [66].

2.4.2. HAMP gene mutation

Genetic alterations in the HAMP gene compromise hepcidin function. The HAMP gene on chromosome 19 contains exon 3, the final exon that encodes the active peptide, and which has the largest polymorphic region of the gene [1, 2, 18]. Polymorphisms present in the HAMP gene are less frequent than polymorphisms in the HFE gene. Currently, about 16 different types of Single Nucleotide Polymorphism (SNP) polymorphism are described, but only a few are of clinical importance, since they are present in haemochromatosis. The first genetic alteration in the HAMP gene was identified using microsatellite marker probes in a region of 2.7 cm in the 19q13. A homozygous region with two mutations was identified (93delG e 166C → T) in two families that presented iron overload in the first decades of life, characterizing juvenile haemochromatosis type 2B [67].

In the mutation minisense homozygosis c.233G>A occurs the exchange of amino acids in the biologically active peptide, with a homozygous substitution of amino acids in the coding region, C78, by a tyrosine, C78T. This mutation disturbs one of the eight cysteines that make the bisulfite bonds necessary for the binding of hepcidin to ferroportin, causing the accumulation

and exaggerated absorption of iron [68]. The C70R mutation also causes malformation in one of the bisulfite bridges of hepcidin cysteines. The amino acid cysteine is exchanged for an arginine, this exchange of neutral amino acid by an acidic amino acid interrupts the formation of the bisulfite bridge between the third and the sixth cysteine in the mature hepcidin peptide [69].

The C → T transition at position 166 in exon 3 of the HAMP (166C-T) cDNA, changes an arginine at position 56 to a stop codon (R56X), 193A → T. The amino acid change of R56X occurs at a region of the mature peptide, at amino acid residues 55–59. This region is a cleavage site for the pro-hormone convertase enzyme to recognize and produce a pro-hepcidin of 64 amino acids. With the change in the peptide chain, the convertase enzyme generates a truncated pro-hepcidin without all the mature sequences. Thus, truncated hepcidin does not bind to ferroportin, causing iron overload from infancy. In contrast, the deletion of a guanine in exon 2 at position 93 of the cDNA (93delG) results in a mutated RNA, which after translation yields a mature peptide of 179 amino acids, which is unstable and inefficient [67, 70].

The deletion Met50del IVS2þ1 (−G) in frameshift of exon 2, is a four nucleotide ATGG deletion that causes a shift of reading frames, disrupting expression of the active peptide, which is encoded by exon 3. The mutation suppresses the last exon 2 codon (Met50) and the first base of the intron 2 binding site (IVS + 1 (−G)). This change increases the reading frame beyond the end of the normal transcript. Another mutation, G71D, alters the charge of amino acid 71, which is between the third and fourth cysteines, at amino acids residues 70 and 72 in leaf β of the peptide, preventing binding with ferroportin [71].

The HAMP-G71D variant in association with the HFE-H63D variant patients with sickle cell disease increases the iron overload [72]. In the Spanish population, the HAMP-G71D variant appearing 1/100 diagnosed cases of haemochromatosis, is not attributed to iron overload [73]. The transition polymorphism G → A at the +14 position of the 5′-UTR region results in a new initiation codon at the +14 position of the 5′-UTR, inducing a change in the reading frame, yielding a new abnormal protein. The transition generates an unstable protein, which is degraded shortly after the translation of the messenger RNA [74].

The TG haplotype of the HAMP gene, caused by the binding of polymorphisms nc-1010C > T and nc-582A > G, is more frequent in H63D HFE individuals, with serum ferritin levels above 300 μg/L [75]. The association of HAMP and HFE gene polymorphisms is frequent. In some cases, there is a mixed clinical condition, with onset of iron accumulation in childhood and severe organ involvement in adult life and vulnerability to infections. The variants C-153C > T and C, 582A > G, reduce the expression of hepcidin, but the mature peptide mechanism of action remains the same, without increasing ferritin and transferrin saturation [76].

Genetic alterations in the HAMP gene may cause a defective or truncated hepcidin, making its mechanism of action impossible. Iron accumulation, as well as increased ferritin enables the development and pathogenesis of certain diseases, such as type 2 diabetes mellitus, coronary disease, increases plasma viremia of HIV, HCV and HBV, generates reactive oxygen species that damage the tissues and cell membranes [77, 78]. The plasma concentration of hepcidin influences the development of neurodegenerative diseases, such as Parkinson's disease,

Alzheimer's disease, multiple sclerosis and in dyslipidemias, some authors attribute this fact, the constant production of IL-6 and the activation of the STAT/Janus kinase pathway [79].

2.4.3. Haemojuvelin gene mutation

The HJV gene or HFE2 encodes the haemojuvelin protein, present on chromosome 1q21. The haemojuvelin is expressed in the liver, heart and skeletal muscle. The haemojuvelin transcript has five isoforms, with the longest protein having 426 amino acids [80]. Haemojuvelin is a co-receptor of the BMP protein, analogous to the Repulsive Guidance Molecules (RGM) family, which binds to BMP receptors to increase SMAD phosphorylation [81, 82]. Genetic alterations in the HFE2 gene make it impossible to transcribe hepcidin. In this way, parenchymal and tissue iron accumulation occurs in the first decades of life and is classified as juvenile haemochromatosis type-2A [83].

Currently, there are 43 HIV mutations identified that cause juvenile haemochromatosis, G320V being the most frequent, with the exchange of a glutamate by a valine [80, 84]. In the Greek population, this polymorphism is frequent in homozygosis, and in the Canadian population, this SNP has been reported in heterozygosity with R326X. Hyperferritinemia and transferrin saturation occur above 45%. More severe cases are found in heterozygosity with the HFE gene, for the polymorphisms C282Y and H63D [85]. Heterozygous patients for G320V and C282Y/H63D have iron and ferritin content 6–8 times higher than patients homozygous for G320V [86, 87]. The severity of the disease can be attributed to the fact that two signalling pathways of the HAMP gene for hepcidin transcription are defective, with the activation of BMP [88–90]. Several different polymorphisms may be involved in iron overload Of hemochromatosis, making difficult the correct diagnosis and precocious. Since each change It presents different clinical characteristics and the need for differentiated treatments, since Iron overload is associated with increased risk of early-onset liver cancer [91–93].

2.4.4. Transferrina (TFR2) gene mutation

The gene encoding the transferrin receptor type 2 (TFR2) is located on chromosome 7. The transferrin receptor 2 is a member of the TFR family, homologous to TFR1. TFR2 in the liver is considered as an iron sensor and activator of the hepcidin pathway. TFR is expressed in the erythroid tissue, where it is a component of the Erythropoietin (EPO) receptor, necessary for effective erythropoiesis to occur. The homozygous nonsense mutation at the 7q22 locus of the TFR2 gene leads to a rare form of iron overload, hereditary haemochromatosis type 3 [94, 95]. Sequencing of the coding region in exon 6 of the gene detected a transversion C → G at position 750 of the cDNA sequence that substitutes a tyrosine (TAC) for a stop sign (TAG) at residue 250 of the transcript (Y250X), giving a defective receptor [94].

Type 3 haemochromatosis is considered as an intermediary between haemochromatosis HFE and haemochromatosis 2. The first signs of iron accumulation occur after 30 years, with heterozygosis for HFE, but some more severe cases, the onset of signs and symptoms begins in childhood. Cardiac disorders and endocrine dysfunctions are less frequent than in juvenile haemochromatosis, with low serum concentrations of hepcidin [96, 97]. TRF2 acts the transcription in the HAMP gene through its interaction with the HFE protein [98]. Defective

formation in the HFE and TFr2 proteins causes an overload of iron [99, 100]. Some authors suggest that TFR2 is required for phosphorylation of BMP6 in response to iron levels, playing a prominent role in the signalling pathway of the HAMP gene and the HFE protein being a contributor to the transcription of the HAMP gene [101, 102].

2.4.5. Ferroportin gene mutation

On chromosome 2, the SLC40A1 gene is located. Alterations in this gene give rise to the disease of ferroportina, an autosomal dominant disease, with two forms, classic form or type A and the non-classic form or type B. It has a global geographic distribution with heterogeneous phenotypic, clinical and genetic characteristics. Although rare, type 4 haemochromatosis is the second major form of iron overload after haemochromatosis HFE. Patients present hyperferritinemia, normal or low transferrin saturation and increased iron load on the Kupffer cells, liver and spleen [103, 104].

The classical form of the disease is usually asymptomatic without tissue damage and without other complications in young life. With age, damage to the liver and pancreas may occur with the development of fibrosis and low haemoglobin concentration [105]. Mutations A77D, D157G, V162del, N174I, Q182H, Q248H and G323V are most frequent in the European, Asian, Indian, Australian and African populations [106, 107]. The genotype Q248H is associated with hyperferritinemia, being frequent in the African population [108, 109]. Classical mutations D157N, D181V, G80V, Q182H, R489K and V162del, alter the folding of ferroportin, damaging the export of iron, and with intracellular accumulation of ferritin in monocytes [107, 110].

The non-classical or B form of ferroportin is more rare and similar to haemochromatosis type 1. Mutations N144H, Y64N, C326Y/S, S338R, Y501C are related to this disease phenotype [107]. Genotypes Y64N, V72F and Y501C confer resistance to hepcidin. These mutations alter the thiol form at residue 326 of the protein, which is essential for the binding of hepcidin to ferroportin, resulting in more severe iron overload at an early age [109]. The S209L mutation described in the Chinese population showed resistance and hepcidin [110]. *In vitro* experiments have demonstrated that the N144D/T and Y64N mutations prevent the internalization of the hepcidin-ferroportin complex, avoiding the ubiquitination of ferroportin within the lysosomal endosomes [111]. The differential diagnosis between types of haemochromatosis type 4 should be made through molecular tests and the patients' clinic [112].

2.4.6. Hereditary a(hypo)ceruloplasminemia and hyperferritinemia

Ceruloplasmin exhibits ferroxidase activity, a condition in which ferrous iron is oxidized to ferric iron to bind to transferrin. Genetic alterations inhibit ferroxidase activity or preclude complete transcription of the enzyme [99]. Aceruloplasminemia is an autosomal recessive disease characterized by progressive neurodegeneration of the retina and basal ganglia associated with inherited mutations. The disease reaches the central nervous system, with increased lipid peroxidation and ataxia syndrome. Other parenchymal tissues, liver and pancreas are also affected [113, 114].

Changes in chromosome 3q24-q25, give rise to the truncated formation of a premature stop codon. The activity of ceruloplasmin ferroxidase is dependent on a trinuclear copper pool, which is encoded by exon 18. The Y356H, R701W and G876A variants are associated with impaired ferroxidase activity of the enzyme, as they generate proteins with altered iron binding sites and

changes in the copper cluster. Studies show the association of these variants with Alzheimer's disease, Parkinson's disease and the development of type 1 diabetes with the appearance of signs and symptoms between 30 and 35 years old, with decreased iron content and increased ferritin (hyperferritinemia) [115–117].

Another hereditary alteration that causes hyperferritinemia, is mutation in the L-ferritin ferritin light chain (FTL) gene located on chromosome 19q13 [118]. The hereditary hyperferritinemia-cataract syndrome (HHCS) is caused by heterogeneous mutations in the iron responsive element (IRE) in the 5′ region of L-ferritin mRNA that reduce the binding affinity of iron-responsive proteins (IRPs) to IREs and decrease the negative control of L-ferritin. This leads to the constitutive positive regulation of the L-chain synthesis of ferritin that characterizes HHCS. Serum ferritin concentrations are high and constant and transferrin saturation is normal. Patients with HHCS do not develop iron overload. The deposition of L-ferritin in the ocular lens causes bilateral cataract at an early age [119]. The 51G-C mutation in the UTR region (ITR) of the FTL gene induces a base pair rearrangement in the lateral structure of the IRE, changing the IRE conformation. The phenotype presents with a moderate increase of serum ferritin, and the development of cataracts may occur throughout life in cases of homozygous [120, 121].

Neuroferritinopathy is a rare, late-onset movement disorder characterized by iron and ferritin accumulation in the brain, normal or low levels of serum ferritin and variable clinical characteristics. The mutations 458dupA, 460insA, A96T, 498insTC alter the conformation and structure of ferritin, resulting in long sequences (26–33 amino acids) that are added to the protein [122]. The insertion, 460insA, of an adenine after nucleotide 460 of the FTL gene alters 22 amino acid residues in the C-terminal region, adding four more amino acid residues after translation of the mRNA. This mutation alters the final conformation of the ferritin molecule as it disrupts the D and E helices. These helices are important because they structure hydrophobic channels for iron core formation in ferritin [123]. Several pathologies such as diabetes, Parkinsonism and dementia are associated with hyperferritinemia [124].

2.5. Genetic changes related to iron deficiency

Genetic changes related to iron deficiency anaemia involve polymorphisms and mutations in the TMPRSS6 gene, present in the chromosome 22 (22q12.3-13.1). This gene encodes the transmembrane protein matritase-2, which cleaves the haemojuvelin, preventing the activation of the BMP/SMAD pathway in the HAMP gene. Genetic alterations involving this gene characterize a phenotype of iron resistance. The iron-refractory iron deficiency anaemia (IRIDA) is an autosomal recessive disease characterized by microcytic and hypochromic congenital anaemia, low transferrin saturation, low iron concentration, elevated serum ferritin and excess hepcidin in serum, plasma and urine, without a therapeutic response to iron replacement and erythropoietin [125, 126]. Excess hepcidin in IRIDA may explain: (1) the development of systemic iron deficiency, preventing the absorption of iron into duodenal enterocytes and the movement of iron stores, (2) the inability to obtain a haematological response to oral therapies with iron and (3) slow and incomplete use of parent iron formulations, consisting of ferro-carbohydrate complexes that require macrophage processing to be available in erythropoiesis [125].

The polymorphisms of the SNPs type, the TMPRSS6 gene, with the greatest clinical impact are K225E, K253E, G228D, R446W, V736A and V795I. In the rs855791 polymorphism, a substitution

of the amino acid alanine for a valine at position 736, in the serine protease domain of matriptase-2 (p.Ala736Val) occurs, is the most frequent, being responsible for the picture observed in IRIDA, with the T allele being associated with decreased iron reserves in men. Mice with Tmprss6−/− phenotype presented severe alopecia and severe iron deficiency anaemia with elevated serum levels of hepcidin and decreased expression of ferroportin [6, 127, 128].

The variant p.V736A influences the susceptibility to hepatic iron accumulation in patients with thalassemia, and the risk allele is 736 (A) [129]. The T287N variant contributes to a history of microcytic anaemia and increases in hepcidin levels due to the inactivation of haemojuvelin cleavage in soluble haemojuvelin, thereby preventing haemojuvelin and hepcidin [130, 131]. Thus, the BMP path remains enabled [132, 133]. The mutation c.1113G >A disrupts the splicing process of mRNA, causing decrease of plasma protein matriptase-2 [133]. The H448R variant and the A719T and V795I polymorphisms are attributed to partial response to treatment with oral iron replacement in iron deficiency anaemia. The lack of response to treatment with oral iron is attributed to high concentration of hepcidin [134].

2.6. Therapeutic targets

The serum concentration of hepcidin determines the pathological state of many diseases-related to iron metabolism, since the modulation of the hepcidin/ferroportin axis has been studied as one of the main therapeutic targets for the treatment of diseases related to serum iron content, among them, haemochromatosis. Currently, the treatment of the hereditary hyperferritinemia (HH) is based on iron chelators and phlebotomy in patients; however, these are not effective treatments, bringing damage to the patient over a long time [135]. However, a stimulus for increased production of hepcidin or a hepcidin analogue may aid in the treatment of hereditary haemochromatosis. On the other hand, IRIDA and iron deficiency anaemia stand out as the main iron-deficient diseases. In this sense, the use of hepcidin inhibitors or TMPRSS6 analogues is a good deal for the treatment of these diseases [136].

2.7. Hepcidin agonist

Hepcidin agonist drugs are being investigated for the treatment of diseases related to lack or partial deficiency of hepcidin. It is a therapeutic alternative for diseases related to iron overload. PR65, a prototype of hepcidin, had its structure developed based on the N-terminal portion of the hepcidin molecule, which contains the amino acid residues, DTHFPICIF, required to bind to ferroportin and promote its ubiquitination, but did not exhibit activity *in vivo*. After molecular improvement of the PR65 prototype, a mini-hepcidin was synthesized. PR65 was administered twice weekly, subcutaneously in rats *knockout* (*HAMP*−/−), which presented iron overload and haemochromatosis phenotype. Iron deficiency was observed in cardiac and hepatic tissue, intestinal adsorption and retention of iron in the spleen and duodenum. When high doses of PR65 were administered in rats, anaemia was observed in these animals. The mini-hepcidins may be used in the auxiliary treatment of β-thalassemia and in policitemia vera [137–140].

Another alternative for the treatment of iron overload is an inhibitor of TMPRSS6, to increase serum hepcidin levels [141]. The use of interfering RNA (siRNA) in suppression of the TMPRSS6 gene in rats with β-thalassemia increased the concentration of hepcidin [142, 143].

Another alternative in the study is the modulation of the BMP/SMAD pathway, with exogenous treatment of BMP6, stimulating the transcription of the HAMP gene [144].

2.7.1. Hepcidin antagonists

Inhibition of the JAK/STAT pathway is used as a therapeutic target in order to reduce the serum hepcidin content with the use of anti-IL-6 receptor antibodies. Studies in animals with anaemia using the antibody, tocilizumab, demonstrated that protein C-reactive (PCR) levels and anaemia improved significantly within 1 week of treatment [145–147]. Siltuximab and infliximab (anti-TNF-α) antibodies have shown good results in the treatment of chronic disease anaemia due to blockage of the JAK/STAT3 pathway, increase in haemoglobin (15 g/dL) and decrease in hepcidin [147, 148]. Inhibition of the STAT3 pathway was also observed using AG490 (Calbiochem), and the peptide PpYLKTK, curcumin [149, 150].

Another alternative for the serum decrease in hepcidin is the intravenous use of *Spiegel mer* lexaptepid. *Spiegelmers* are synthetic oligonucleotides binding to molecular targets. Currently, three oligonucleotides are in clinical study, NOX-E36 (anti-CCL2), NOX-A12 (anti-CXCL12) and NOX-H94 (anti-hepcidin). *Spiegelmer* NOX-H94 blocks the binding of hepcidin to ferroportin, preventing its proteolytic ubiquitination, and may be used in the treatment of IRIDA and in diseases with increased hepcidin and increased IL-6. Studies in monkeys have shown improvement in anaemia and increased serum iron concentration [151–154].

The LDN-193189 is an inhibitor of BMP type I receptor, attenuates BMP/SMAD signalling in hepatic tissue and reduces HAMP gene [155]. The administration of LDN193189 facilitated the movement of iron stores in rats with anaemia of inflammation, with increased haemoglobin, erythropoiesis and reticulocytosis [156, 157]. The inibition of hepcidin synthesis may occur by blocking the ALK2 (activin-like receptor), receptor the BMPs proteins [158]. TP-0184 is an inhibitor of the ALK2 receptor, and in pre-clinical phase studies, it has shown to be a negative regulator of hepcidin expression. The monoclonal antibodies ABT-207 and h5F9-AM8 that inhibit haemojuvelin has been demonstrated to be effective in decreasing the expression of hepcidin in rats. Another treatment alternative to inhibit HAMP gene transcription is the fusion between Hemojuvelin soluble (sHJV) with the Fc portion of an IgG, giving Hemojuvelin Fracion (sHJV.Fc), which binds to BMPr, inhibiting the BMP6/SMAD pathway [136, 159–161].

Fursultiamine and LY2928057 antibodies prevent the interaction between ferroportin-hepcidin by sequestering the Cys326-HS residue of ferroportin and blocking the internalization of ferroportin/hepcidin [136, 162]. Anti-hepcidin antibodies efficacy was confirmed in a model of anaemia of inflation induced by *Brucella abortus*, in which hepcidin neutralization occurred and anaemia improved. The humanized antibody, 12B9m, has been shown to be a potent inhibitor of hepcidin [163–165].

The first evidence of heparin's action in controlling hepcidin expression was demonstrated in HepG2 cells and in rats that were given pharmacological doses of heparin. In the study, hepatic hepcidin mRNA expression decreased, from the sequestration of the BMP-6 protein and subsequent phosphorylation of the SMAD1/5/8 complex with reduction of iron concentration in the spleen and increase of serum iron. From these results, structural modifications in the heparin molecule were performed in order to decrease its anticoagulant activity for

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the clinical use of hepcidin elevation diseases. Modified heparins, by oxidation/ reduction, heparins *glycol-split* (*GS-heparin*), or sulfation (*SS-heparin*), demonstrated in clinical trials with little or no toxicity, being potent inhibitors of hepcidin *in vitro* in HepG2 cells and primary hepatocytes, and *in vivo* in rats, without presenting anticoagulant activity [166–169].

Growth differentiation factor 15 (GDF15) was shown to negatively regulate hepcidin mRNA expression in humans [170]. The compound K7174 GDF15 increases synthesis and reduces hepcidin expression in HepG2 cells and in rats [171]. The erythroid Erythroferrone (ERFE) hormone presents suppression activity of hepcidin mRNA in rat model with β-thalassemia [172, 173]. The siRNAs, siHJV, siTRF2 and siHepcidin are the main apostasy for suppression of the HAMP gene with inhibition of hepcidin synthesis [174].

2.8. Conclusion

The homeostasis of iron metabolism involves several signalling pathways and gene regulations. Genetic alterations influence the development of several pathologies involving hepcidin. Currently, screening for the diagnosis and research of polymorphisms and mutations related to iron metabolism is scarce. Genetic diseases with iron overload are diagnosed very late, bringing damage to patients' health. Studies of allelic frequencies in the most diverse populations are still scarce, making it difficult to trace genetic polymorphisms and phenotypic changes. There are few therapeutic options for the treatment of genetic diseases related to iron metabolism, and are still in clinical research phase II and I. Although these diseases are rare, the diagnosis is underestimated and directly interferes with the treatment of the patient. Epigenetic alterations can affect non-pathogenic polymorphisms by making them harmful; therefore, a complete description of the possible interferents in the genetic polymorphisms and the probable changes that these polymorphisms offer to the human organism are necessary.

Conflict of interests

The authors state that there are no conflicts of interest.

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