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Serum Hepcidin, erythroferrone and soluble transferrin receptor at population level: towards a better definition of some iron related disorders

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Serum Hepcidin, erythroferrone and soluble transferrin receptor at population level: towards a better definition of some iron related disorders

Acaynne Lira Zidanes

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ABSTRACT

Iron is an essential element for all living organisms, and it is involved in a variety of vital functions including cellular energy metabolism, anaerobic respiration, synthesis of hemoglobin and nucleotides. On the other hand, free iron is highly reactive and toxic due to its capability to accept and donates electrons and generate oxygen-derived free radicals (Reactive Oxygen Species-ROS). Thus, iron content in the body needs to be tightly regulated. Hepcidin is the principal iron homeostasis regulator by controlling plasma iron levels and total body iron, and its alteration can lead either functional iron deficiency (high hepcidin levels) or iron overload (low hepcidin levels). Erythroferrone (ERFE) is the main erythroid regulator of hepcidin. Increased ERFE levels suppress hepcidin synthesis, thereby mobilizing cellular iron stores for use in heme and hemoglobin synthesis. In ineffective erythropoiesis, pathological overproduction of ERFE by an expanded population of erythroblasts suppresses hepcidin and causes iron overload. Soluble transferrin receptor (sTfR) is a cleaved form of the tissue transferrin receptor 1 (TfR1). sTfR gives information on the functional iron status and its levels are increased in iron deficiency (ID) and when erythropoiesis is stimulated.

The main aim of this study was to investigate iron metabolism determinants at population level by the analysis of serum hepcidin, ERFE and sTfR concentrations in 4644 subjects of the Val Venosta population, a homogeneous population in South Tyrol-Italy, as part of a larger project established in 2011 for the investigation of the genetic basis of common chronic conditions associated with human ageing and their interaction with life-style and environmental factors in the general population of South Tyrol (The CHRIS study).

Serum hepcidin (20, 24 and 25 isoforms), ERFE and sTfR measurement was performed in the Laboratory of Iron Disorders at the LURM (University Laboratory for Medical Research), by means of an LC-MS/MS approach for hepcidin, and ELISA techniques for ERFE and sTfR.

Hepcidin-25 confirmed an age and sex dependent variation, while ERFE showed a less significant age- and gender-dependent variation. Hepcidin-25 correlation

analyses showed that it correlates more with ferritin than with serum iron and transferrin saturation, whereas ERFE showed a negative relationship with hemoglobin and red blood cells. The results here presented, such as the establishment of an updated reference range for hepcidin-25 and ERFE levels and the investigation of hepcidin and ERFE variation in different conditions, could have clinical applications, as well as underling the usefulness of these emergent biomarkers for the assessment of human iron status in diagnostic approaches.

RIASSUNTO

Il ferro è un elemento essenziale per tutti gli organismi viventi ed è coinvolto in una varietà di funzioni vitali, tra cui il metabolismo energetico cellulare, la respirazione anaerobica, la sintesi di emoglobina e nucleotidi. Tuttavia, il ferro libero è altamente reattivo e tossico grazie alla sua capacità di accettare e donare elettroni e generare radicali liberi derivati dall'ossigeno (Reactive Oxygen Species-ROS). Pertanto, il contenuto di ferro nel corpo deve essere strettamente regolato. L'epcidina è il principale regolatore dell'omeostasi del ferro, controlla i livelli plasmatici di ferro e il ferro corporeo totale e la sua alterazione può portare sia a carenza di ferro (alti livelli di epcidina) sia a sovraccarico di ferro (bassi livelli di epcidina). L'eritroferrone (ERFE) è il principale regolatore eritroide dell'epcidina. L'aumento dei livelli di ERFE sopprime la sintesi di epcidina, mobilitando così le riserve di ferro cellulare per l'uso nella sintesi di eme ed emoglobina. Nell'eritropoiesi inefficace, la sovrapproduzione di ERFE da parte di una popolazione espansa di eritroblasti, sopprime l'epcidina e causa il sovraccarico di ferro. Il recettore solubile della transferrina (sTfR) è una forma scissa del recettore 1 della transferrina tissutale (TfR1). sTfR fornisce informazioni sullo stato funzionale del ferro e i suoi livelli sono aumentati in caso di carenza di ferro (ID) e quando viene stimolata l'eritropoiesi.

Lo scopo principale di questo studio era indagare i determinanti del metabolismo del ferro a livello di popolazione, mediante l'analisi delle concentrazioni sieriche di epcidina, ERFE e sTfR in 4644 soggetti della popolazione della Val Venosta, una popolazione omogenea in Alto Adige-Italia, come parte di un progetto più ampio istituito nel 2011 per lo studio delle basi genetiche delle comuni condizioni croniche associate all'invecchiamento umano e della loro interazione con lo stile di vita e fattori ambientali nella popolazione generale dell'Alto Adige (studio CHRIS).

La misurazione dell'epcidina sierica (isoforme 20, 24 e 25), ERFE e sTfR è stata eseguita nel Laboratorio dei Disturbi del Ferro presso il LURM (Laboratorio Universitario di Ricerca Medica), mediante un approccio LC-MS / MS per l'epcidina e tecniche ELISA per ERFE e sTfR.

L'epcidina-25 ha confermato una variazione dipendente dall'età e dal sesso, mentre l'ERFE ha mostrato una variazione meno significativa. Le analisi di correlazione dell'epcidina-25 hanno mostrato che essa si correla di più con la ferritina che con la saturazione sierica di ferro e transferrina, mentre ERFE ha mostrato una relazione negativa con l'emoglobina e i globuli rossi. I risultati qui presentati, come la definizione di un range di riferimento aggiornato per i livelli di epcidina-25 ed ERFE e lo studio della variazione di epcidina ed ERFE in diverse condizioni, potrebbero avere applicazioni cliniche, oltre a sottolineare l'utilità di questi biomarcatori emergenti per la valutazione dello stato del ferro negli approcci diagnostici.

SUMMARY

INTRODUCTION

Iron Metabolism and Hepcidin

Iron is an essential element which plays a central role in cellular energy metabolism, anaerobic respiration, synthesis of hemoglobin and nucleotides. Moreover, iron is involved in many other processes in the body such as oxidative metabolism and cellular immune response [1]. On the other hand, free iron is highly reactive and may be toxic because of its capability to generate oxygen-derived free radicals (Reactive Oxygen Species-ROS). The most potentially dangerous among the ROS is the hydroxyl ion $OH^{\bullet}$, a short-lived but highly reactive free radical, generated through the Fenton reaction $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH^{\bullet}$. Thus, iron content in the body needs to be tightly regulated [2].

Systemic iron homeostasis is highly conservative and, in the adult, the total amount of body iron is about 3-4 g. Under physiological conditions, red blood cells (RBCs) contain the largest proportion of body iron (~ 2 g), and the 20-25 mg of iron needed for daily production of new RBCs derive almost totally from continuous recycling of aged erythrocytes by splenic macrophages [3]. Every day, 1-2 mg/d of iron is absorbed from the diet in order to match the same amount that is lost through skin and mucosal exfoliation, as well as menses in fertile women (Figure 1). It should be noted that the organism does not have an active iron excretion mechanism, thus the control of the duodenal absorption plays a vital role in iron homeostasis [4].

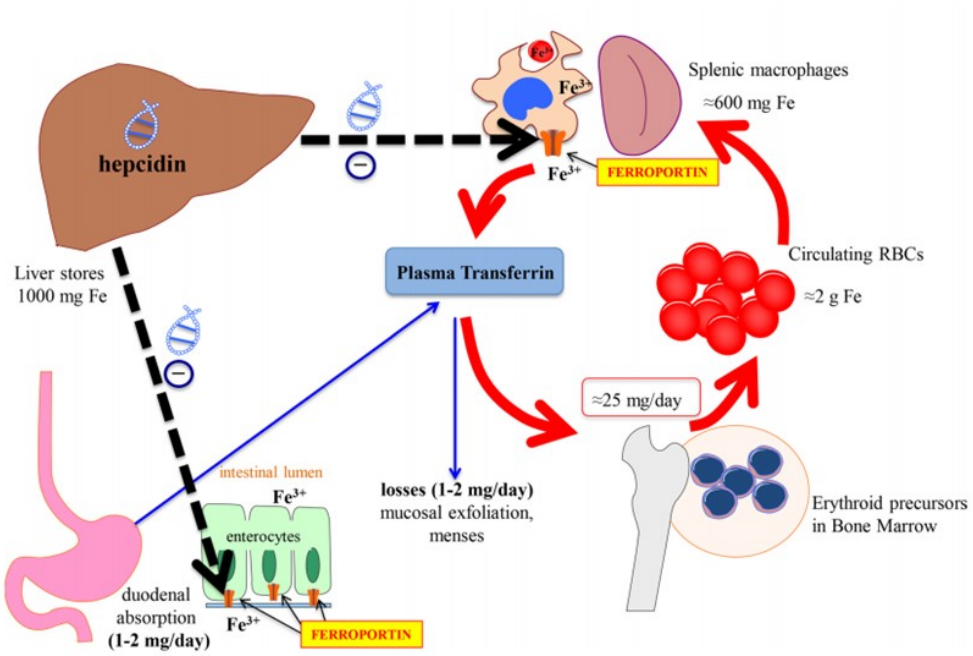


Figure 1: Systemic Iron Homeostasis is highly conservative of total body iron content (3–4 g), through the continuous recycling of iron from the senescent erythrocytes by splenic macrophages, which supplies the 20–25 mg/day of iron needed for bone marrow hematopoiesis (thick red arrows). Total body iron homeostasis is maintained by accurately matching unavoidable daily losses with intestinal absorption of dietary iron, (1–2 mg/day) (thin blue arrows). The master regulator is hepcidin, which neutralize ferroportin (black dotted arrows). [3]

Hepcidin is a hormone synthesized in the liver and its main role is to regulate the plasma pool of iron. Hepcidin acts by controlling iron absorption from the diet through enterocytes, iron recycling from macrophages involved in the destruction of senescent RBCs, and iron mobilization from the deposits (Figure 1). Hepcidin is produced as a full-length 84 amino acids pre-pro-peptide containing a typical NH_2-terminal 24 amino acids signal sequence which is cleaved to generate the intermediate 64 amino acid pro-protein. Pro-hepcidin is transported from cytoplasm into the lumen of endoplasmic reticulum in which the generation of the mature 25 amino acids hepcidin occurs [5]. In addition to hepcidin-25, shorter isoforms exist: hepcidin-24, hepcidin-22 and hepcidin-20, produced by the N-terminal degradation of hepcidin-25 isoform [6, 7]. In healthy individuals, minor isoforms are found at low to negligible levels compared to the bioactive hepcidin-25, but their concentrations can increase in some pathological processes, including chronic kidney disease. Even though functions of smaller isoforms are still unclear, hepcidin-24 seems to be elevated during inflammation [6] and the 20 amino acids

isoform has been shown to have an antimicrobial activity. The measurement of hepcidin is a promising tool for disorders of iron homeostasis [7, 8]

Hepcidin negatively regulate the iron homeostasis by binding to ferroportin, the cell membrane channel responsible for the efflux of iron from enterocytes, macrophages and hepatocytes into the plasma. After binding to ferroportin, hepcidin causes the occlusion of the channel [9] and subsequent ubiquitination and degradation into lysosomes [10].

The currently known mechanisms to regulate hepcidin are at transcriptional level, acting on the gene that encodes hepcidin (*HAMP*), in response to different stimuli (Figure 2 and Figure 3).

An important factor that regulates hepcidin is plasma iron levels. In normal conditions, the level of circulating iron is proportional to the amount of hepcidin produced by hepatocytes [11, 12]. An increase in the amount of iron induces *HAMP* transcription via BMP/SMAD pathway. Circulating iron-bound transferrin (Tf-$2Fe^{3+}$) is sensed in the liver by a complex molecular machinery, including, among others TfR1, TfR2 and the hemochromatosis protein (HFE) [13]. Increased diferric transferrin seems to displace the HFE protein from TfR1, enabling its interaction with TfR2, which in turn stimulates ALK3, the type 1 Bone Morphogenetic Protein (BMP) receptor (BMPR1). Of note, diferric transferrin also induces the expression of BMP6 and, likely of BMP2 (possibly forming a BMP6-BMP2 heterodimer) by liver sinusoidal endothelial cells (LSEC), which in turn stimulate hepcidin production through binding to type 1/type 2 receptor (BMPR1 and BMPR2). BMPR1/BMPR2 complexes include also a co-receptor, hemojuvelin (HJV), and a facilitator, neogenin (NEO). HJV is a glycosylphosphatidylinositol-linked membrane protein, indispensable to proceed with the cascade. This complex is responsible for the phosphorylation of intracellular SMAD1, SMAD5 and SMAD8, which after binding to the SMAD4 in the cytoplasm, can enter the nucleus and trigger the *HAMP* transcription. A comprehensive and updated view of molecular mechanisms regulating hepcidin production is reported elsewhere [11]. Finally, the iron-BMP signaling pathway is negatively regulated by the serine protease

matriptase-2 (MT-2 or TMPRSS6). TMPRSS6 works by cleaving and inactivating the co-receptor HJV, preventing the BMP complex activation [14] (Figure 2).

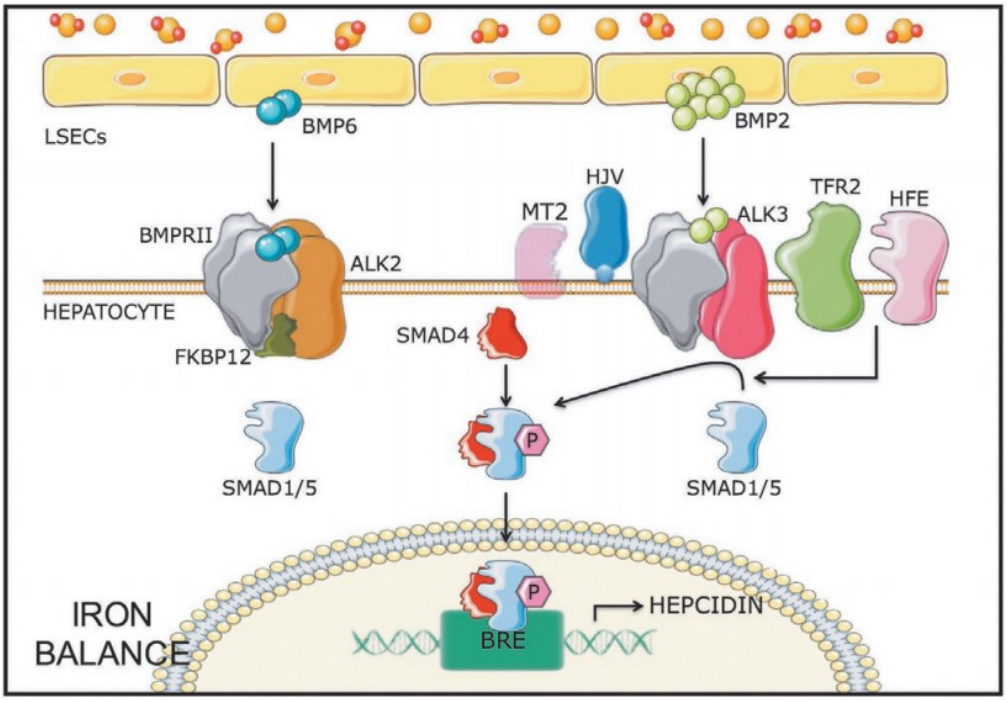


Figure 2: Scheme of hepcidin synthesis regulation in normal iron balance. Two branches of the BMP-SMAD pathway are represented in this figure: one activated by ALK2, that signals through BMP6 and is inhibited by FKBP12; the other, ALK3-mediated, is activated by BMP2 and positively modulated by the BMP coreceptor hemojuvelin (HJV), the second transferrin receptor (TFR2) and the MHC-class I molecule HFE. [15]

Erythropoiesis, as well as hypoxia, can reduce hepcidin expression, with the benefit of increasing iron absorption. Hypoxia can be a consequence of anemia and increases erythropoietic activity through the stimulation of erythropoietin (EPO). Several studies have reported the ability of some erythroid-derived proteins to suppress hepcidin, such as the growth differentiation factor 15 (GDF15), twisted gastrulation BMP signaling modulator (TWSG1) and erythroferrone (ERFE) as possible candidates for this erythroid regulation of hepcidin [16] (Figure 3C).

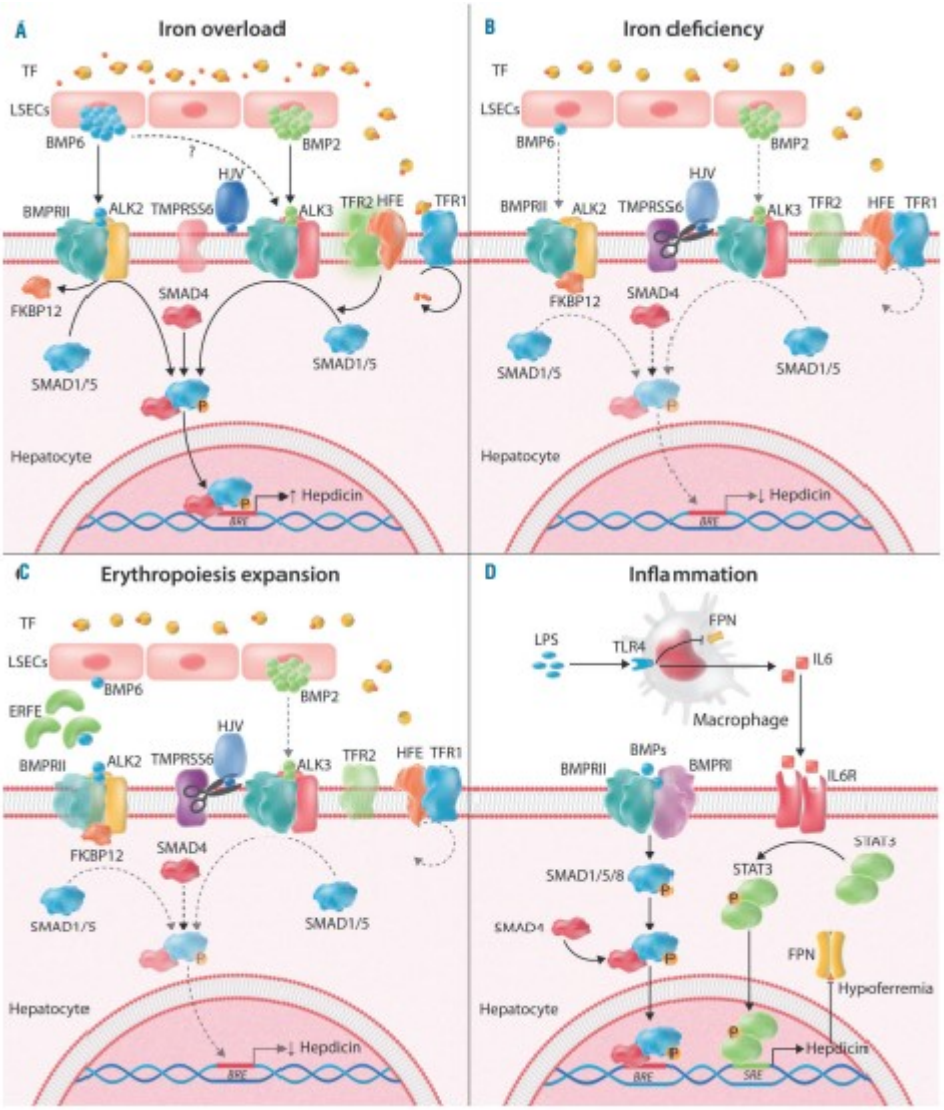


Figure 3: The regulation of hepcidin expression in specific conditions: (A) iron overload, (B) iron deficiency, (C) erythropoiesis expansion and (D) inflammation. [11]

Other conditions that regulate hepcidin expression are chronic infections and inflammatory diseases (Figure 4). Indeed, hepcidin levels turned to be hundred-times higher in patients suffering from inflammatory conditions than in healthy individuals [5, 17, 18]. The link between inflammation and increased hepcidin level in the liver seems to be related to interleukine-1β (IL-1β), IL-6 and IL-22 that act on *HAMP* gene through SMAD/STAT3 pathway (Figure 3D).

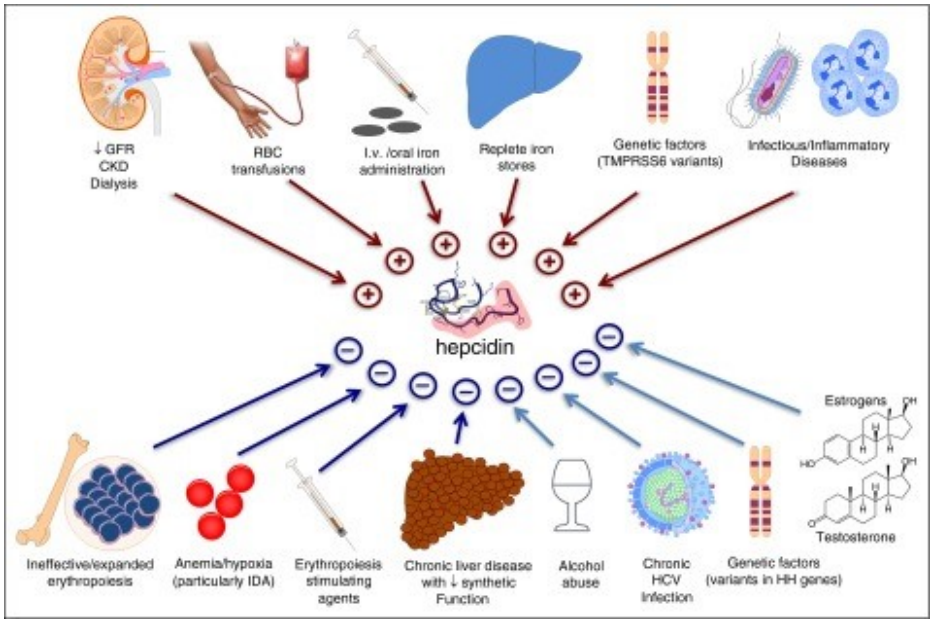


Figure 4: Clinical conditions known to influence circulating hepcidin. Clinically relevant conditions include chronic kidney disease (CKD), RBC transfusions, iron administration, replete iron stores, TMPRSS6 variants (the gene encoding for matriptase-2), infections/inflammatory disorders, ineffective erythropoiesis, hypoxia, administration of erythropoietic stimulating agents, chronic liver diseases, alcohol abuse, HCV infection, hemochromatosis-related mutations and administration of the sex hormones testosterone and estrogens. [7]

Mutations on genes encoding proteins involved in the regulation of hepcidin expression, or in the gene encoding hepcidin itself, may result either in hepcidin deficiency, with consequent iron overload, or in hepcidin excess, causing iron deficiency [19].

Mutations on *HFE*, *TfR2*, *HJV*, and *HAMP* gene cause Hemochromatosis (H), a genetic disease characterized by inappropriately high iron absorption, elevated transferrin saturation and serum ferritin, inappropriately low hepcidin expression and iron overload [13, 20]. Table 1 summarizes all types of H. Depending on the timing, severity, and extent of iron loading, iron-induced tissue injuries include hepatic fibrosis and cirrhosis, endocrinopathies such as diabetes, and cardiomyopathy [21]. These forms of H have similar underlying pathophysiology, with phenotypic differences most likely due to the degree of impairment in the pathways controlling hepatic hepcidin production [22]. More rarely mutations affect ferroportin gene (*SLC40A1*), causing either iron accumulation in Kupffer cells and macrophages in the early stages and a late accumulation in hepatocytes (when the mutation affects the iron transport ability of ferroportin), or a constitutive

iron release from macrophages and enterocytes, thus giving a phenotype similar to H (when the mutation affects the ferroportin sensitivity to hepcidin-mediated degradation) [23].

Protein	Gene	Type of H	Frequency of pathogenic mutations	Inheritance
HFE	*HFE*	Type 1	Frequent	A.R
Hemojuvelin	*HJV*	Type 2A	Rare	A.R
Hepcidin	*HAMP*	Type 2B	Very rare	A.R
Transferrin receptor 2	*TfR2*	Type 3	Very rare	A.R
Ferroportin*	*SLC40A1*	Ferroportin Disease (also called type 4)	Rare	A.D

*Table 1: A summary of four types of hemochromatosis. (A.R: autosomal recessive; A.D: autosomal dominant; * Gain of function mutations on SLC40A1 gene can lead to hepcidin resistance and a clinical picture similar to hemochromatosis. On the other hand, Loss of Function mutations lead to a distinct disorder (Ferroportin Disease), characterized by normal transferrin saturation and iron overload predominantly in liver macrophages (Kupffer cells) rather than in hepatocytes.*

Mutations on *TMPRSS6* gene, encoding the liver expressed hepcidin inhibitor serine protease matriptase-2, cause iron refractory iron deficiency anemia (IRIDA), a type of anemia that is poorly responsive to oral iron administration and only partially response to parenteral iron [24]. IRIDA is characterized by hypochromic, microcytic anemia, low transferrin saturation, intestinal iron malabsorption and high hepcidin levels [25, 26].

Erythroferrone: an erythroid regulator of iron homeostasis

In view of the central role of hepcidin in iron regulation, it was hypothesized that putative erythroid regulators could facilitate iron delivery to the bone marrow by decreasing hepcidin concentrations, therefore allowing increased iron absorption and the release of iron from stores into blood plasma [27]. Indeed, erythroid regulators of iron homeostasis could work as mediators of appropriate responses to hemorrhage, anemia, hypoxia or erythropoietin administration (physiologic

erythroid regulators) [16]. In contrast to their adaptive role in the recovery from blood loss, erythroid regulators may act pathologically to mediate iron overload in inherited anemias with ineffective erythropoiesis such as β-thalassemia and congenital dyserythropoietic anemia (pathological erythroid regulators) [27].

As mentioned before, different factors were predicted to act as erythroid regulator of hepcidin, such as GDF15, TWSG1 and ERFE. However, only erythroferrone fits the description of both physiological and pathological erythroid regulator [16].

Erythroferrone is a hormone synthesized by erythroblasts in erythropoietic organs (bone marrow and spleen) and a member of the C1q/TNF-related protein (CTRP) family. Human ERFE is encoded by a ~10 kb gene divided into 8 exons and located on chromosome 2. Erythroferrone contains a 28 amino acid signal sequence followed by a 326 amino acid mature protein [28, 29].

Experiments in mice first showed the role of ERFE as a mediator of the response to erythropoietic stress [16, 27, 30]. Thanks to the high level of similarity between human and mouse ERFE (71% identity, 77% similarity) it was possible to obtain a correlation between mice studies and studies of human ERFE responses [31]. Similarly to what happened in phlebotomized mice, human blood donors responded to the loss of 2 units of packed erythrocytes (about 400–450 ml) by increasing serum ERFE concentrations (lasting at least 2 weeks) and by the simultaneous decrease of serum hepcidin concentrations, with a return to baseline in both hormones 16 weeks later. Four human subjects underwent EPO administration which caused a rapid rise in serum ERFE with a peak 1–2 days later, and a corresponding decrease in serum hepcidin concentrations [16]. Non transfused patients with β-thalassemia showed a profound increment in serum ERFE and also patients with β-thalassemia analyzed before their regular transfusions. On the other hand, serum ERFE was only mildly increased in the week after RBC transfusions in transfusion-dependent β-thalassemia patients [16]. These studies indicate that there is an inverse correlation between serum ERFE concentrations and serum hepcidin concentrations, so that patients with non-transfusion dependent β-thalassemia (NTDT) had the highest levels of serum ERFE and the lowest hepcidin concentrations. Collectively, these studies indicate that ERFE is a strong candidate for being the principal erythroid

regulator of iron and hepcidin in humans [16]. As for other anemias, ERFE is increased in patients with anemia of chronic kidney disease, and also in end-stage kidney disease after treatment with exogenous EPO, suggesting that ERFE responds appropriately to endogenous and exogenous EPO [32]. Figure 5 summarizes the physiological and pathological roles of ERFE proposed by Tomas Ganz [16].

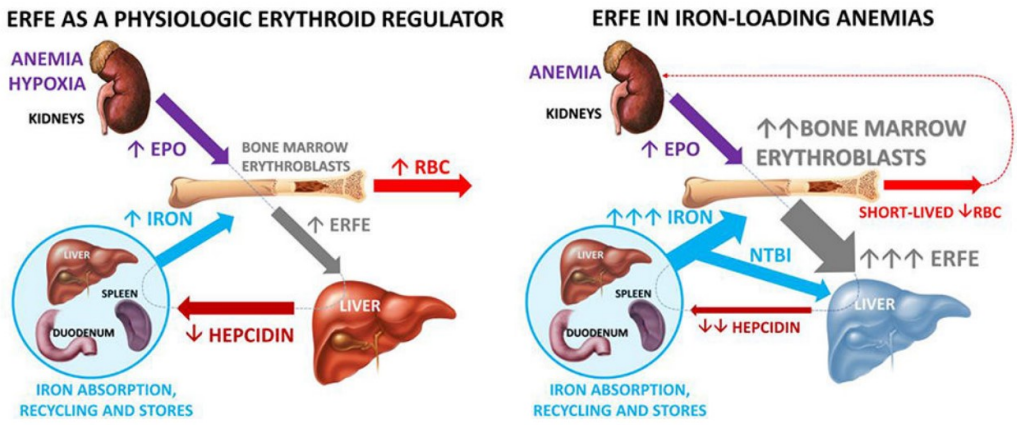


Figure 5: Proposed roles of ERFE in matching iron supply to erythropoietic demand during stress erythropoiesis, such as after acute blood loss or acute hemolysis (left panel), contrasted with the pathological role of ERFE in iron-loading anemias with ineffective erythropoiesis, exemplified by β-thalassemia intermedia (right panel). [16]

Once erythroferrone was discovered as an erythroid regulator essential for hepcidin suppression, the scientific community started to investigate its mechanism of action. Early studies indicated the possibility that hepcidin suppression by ERFE is independent of the BMP pathway [27]. However, further studies demonstrated that ERFE suppressed Smad1/5 phosphorylation in primary murine hepatocytes and in the Hep3B hepatocytic cell line, and that hepcidin suppression by EPO was dependent on intact Smad1/5 signaling in mice. When Smad1 and Smad5 were ablated in primary murine hepatocytes or in Hep3B cells, ERFE could no longer suppress hepcidin mRNA. These studies indicate that hepcidin suppression induced by ERFE is at least in part mediated by the BMP pathway [33]. Other studies have investigated the role of matriptase-2 in the ERFE signaling pathway. Guo and Nai, in their independent researches, showed that $Hbb^{th3/+}$ mice models of thalassemia intermedia with inactivated Tmprss6 had high hepcidin levels, despite high serum EPO concentrations [34, 35]. Successively, Nai and colleagues reported that patients with IRIDA were resistant to treatment with EPO, suggesting that EPO

could not be sufficient to suppress hepcidin when the function of matriptase-2 is lost. Nevertheless, the experiments with mice showed that Tmprss6 knockout mice expressed high levels of ERFE, consistent with their anemia and high EPO levels [36]. A potential explanation of these findings is that the effect of ERFE on hepcidin is linearly additive to that of the BMP pathway [27], so that when the BMP pathway is hyperactivated by the loss of inhibitory Tmprss6 activity the effect of ERFE becomes proportionally smaller [16].

Erythroferrone could be considered as a promising target molecule with different therapeutic applications/implications. ERFE antagonists could be useful for the prevention of iron overload in anemias with ineffective erythropoiesis (β-thalassemia, congenital dyserythropoietic anemias, some sideroblastic anemias and pyruvate kinase deficiency). Even when present at pathologically excessive concentrations, ERFE is a subnanomolar hormone thus neutralization by humanized monoclonal antibodies is likely to be feasible with relatively few dosing. On the other side, ERFE analogs (molecules that mimic ERFE activity) would be useful in conditions where hepcidin levels are high, and the iron restriction hampers erythropoiesis (including anemia of inflammation and anemia of chronic kidney disease) [16, 29].

A lot of progress has been made on the knowledge of erythroferrone, however many fundamental questions remain uncertain regarding its mechanism(s) of action. To date no putative receptor for ERFE has been identified and little is known about its signaling pathway. Other important tasks include the standardization of human and mouse ERFE assays, and the exploration of the diagnostic utility of ERFE measurements in different forms of anemia [16]. Studies of ERFE at population level and genome-wide association studies (GWAS), currently never performed, could be important instruments to facilitate rapid progress in this field.

Soluble Transferrin Receptor

Iron transport in the plasma is carried out by transferrin, which donates iron to cells through its interaction with a specific membrane receptor. Two types of transferrin receptor (TfR) have been identified. TfR1 is present on the surface of almost all proliferative cells, with the exception of mature erythrocytes and some other

terminally differentiated cells, and mediates the uptake of iron for the cell cycle. On the contrary, TfR2 is mainly expressed in the liver and its dysfunction is correlated with the type 3 H [37, 38].

TfR1 is a transmembrane glycoprotein composed of a disulfide-bonded homodimer in which subunits are connected by two disulfide bonds at cysteines 89 and 98. Each subunit containing 760 amino acids was found to be divided into three main portions: a large C-terminal extracellular domain of 671 amino acids, a single-pass transmembrane domain of 28 amino acids, and an N-terminal cytoplasmic domain of 61 amino acids (Figure 6) [38]. In humans, transferrin receptor 1 is encoded by *TfR1* gene of about 32 kb, located in chromosome 3 and organized in 19 exons [39]. High levels of TfR1 expression were found in cells with increased proliferation rate, such as erythroid progenitor cells, placental syncytiotrophoblasts, and neoplastic cells [40].

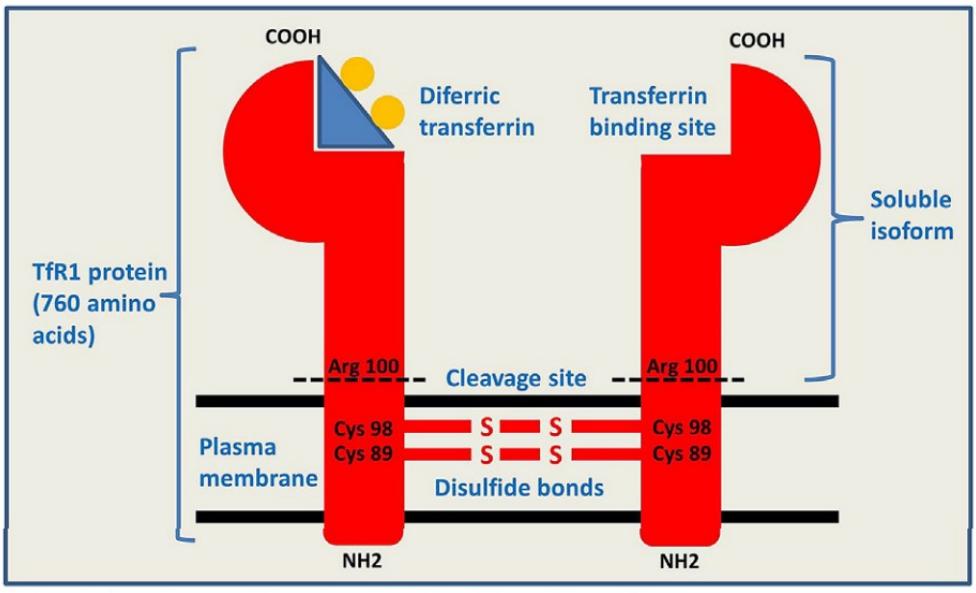


Figure 6: Transferrin receptor 1 (transmembrane and soluble form). [38]

A circulating form of TfR has been found in human as well as in animal serum. Soluble transferrin receptor (sTfR) was identified as a soluble truncated monomer of TfR1, lacking its first 100 amino acids (cytoplasmic and transmembrane domains) (Figure 6), which circulates in the form of a complex of transferrin and its receptor. In plasma, two molecules of ferric iron (Fe^{3+}) are bound by transferrin and delivered into cells after interaction of the holo-transferrin (diferric transferrin)

with the cell surface TfR1 dimer and subsequent internalization of iron-transferrine-TfR1 complex via endocytosis. The release of iron from transferrin occurs after the acidification (pH 5.5) of endosomes (acidosomes) with a subsequent reduction of liberated ferric to ferrous iron (Fe^{2+}). The latter is transported towards the cytosol via DMT1 (Divalent Metal Transporter 1) to be used for metabolic functioning or to be stored in ferritin (Figure 7). After iron release, a portion of the Tf-TfR1 complex returns to the cell surface, where apo-transferrin (free-transferrin) is released again in the plasma. Whereas another portion gives rise to sTfR by proteolysis, mediated by a membrane-associated serine protease, that occurs at the surface of exosomes within the multivesicular body (MVB) prior to exocytosis (Figure 7) [38, 39]. The concentration of sTfR measured in serum appeared to be proportional to the mass of cellular TfR1 [41] and originates mostly from erythroblasts and to a lesser extent from reticulocytes [42].

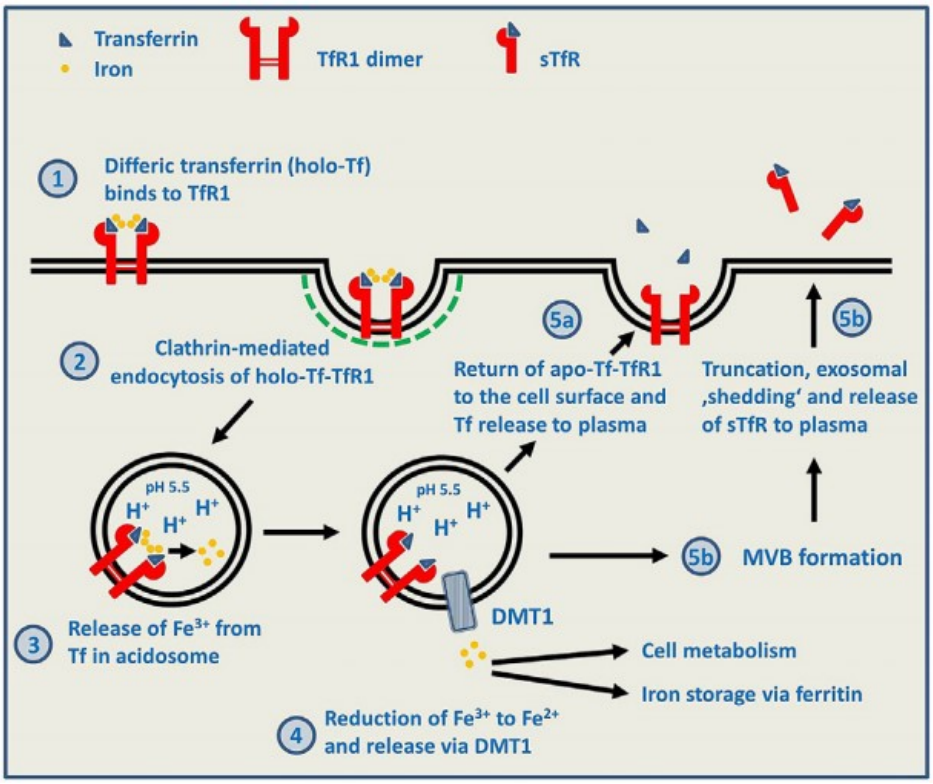


Figure 7: Illustration of the interaction of iron, transferrin, and transferrin receptor in iron metabolism and the generation of soluble transferrin receptor. The putative major route for shedding of sTfR is illustrated in 5b. In contrast, 5a shows the classic recycling pathway of TfR1. [38]

Although the biological function of sTfR remains unexplained, its use as a biomarker of iron status has gained relevance because it gives information on the functional iron status and total erythropoietic activity [43]. sTfR levels are increased in iron deficiency (ID) and when erythropoiesis is stimulated, as in conditions such as congenital dyserythropoietic anemia, (autoimmune) hemolytic anemia, hereditary spherocytosis, sickle cell anemia, thalassemia major or intermedia, megaloblastic or iron deficiency anemia (decreased tissue iron stores), and secondary polycythemia. In contrast, sTfR levels are decreased in conditions characterized by a decreased count of erythroid precursors cells in the bone marrow (erythroid hypoplasia), such as hyper-transfusion, chronic renal failure, aplastic anemia, or after intensive chemotherapy [38]. sTfR is also considered a valuable parameter for the differentiation of iron deficiency anemia (IDA) and anemia of chronic disease (ACD), since sTfR is not affected by inflammation, or is less affected by high exposure to it, unlike ferritin and other standard biochemical markers [37, 38]. Therefore, elevated sTfR levels in patients with ACD or acute inflammation can indicate functional iron deficiency, further supported by its decreased after adequate iron supplementation [44, 45].

Even though some studies tried to establish a reference range for sTfR [46, 47], standardized techniques for its measurement have not been established yet and absolute values depend on the method applied; this issue represents the major obstacle to the routine use of sTfR as a biomarker to assess iron metabolism. Its utility is also affected by the lack of specificity to determine ID in the presence of symptoms of hemolytic and sickle cell anemia since sTfR levels increase in these conditions [37].

The ferritin index is another marker for the assessment of human iron status and is calculated by dividing the value of sTfR by the decimal logarithm of ferritin (ferritin index = sTfR/log ferritin). It covers the full range of iron status from normality to iron deficiency and is considered a good marker for its high sensitivity and specificity. However, ferritin index has a limit linked to the lack of a standardized method for the determination of sTfR [37, 48].

The Cooperative Health Research in South Tyrol Study

Our group took part in a large-scale epidemiological and genetic study named the Cooperative Health Research In South Tyrol (CHRIS) study, that has been previously well described by Pattaro and colleagues [49]. The CHRIS study is population-based and aims at investigating the genetic basis of common chronic conditions associated with human ageing, and their interaction with life-style and environmental factors in the general population of South Tyrol [49]. All adults inhabitants of the middle and upper Vinschgau/Val Venosta, a 70 km long valley in the autonomous province of Bolzano/ South Tyrol, Italy, were invited to take part in the study (Figure 8) [49, 50].

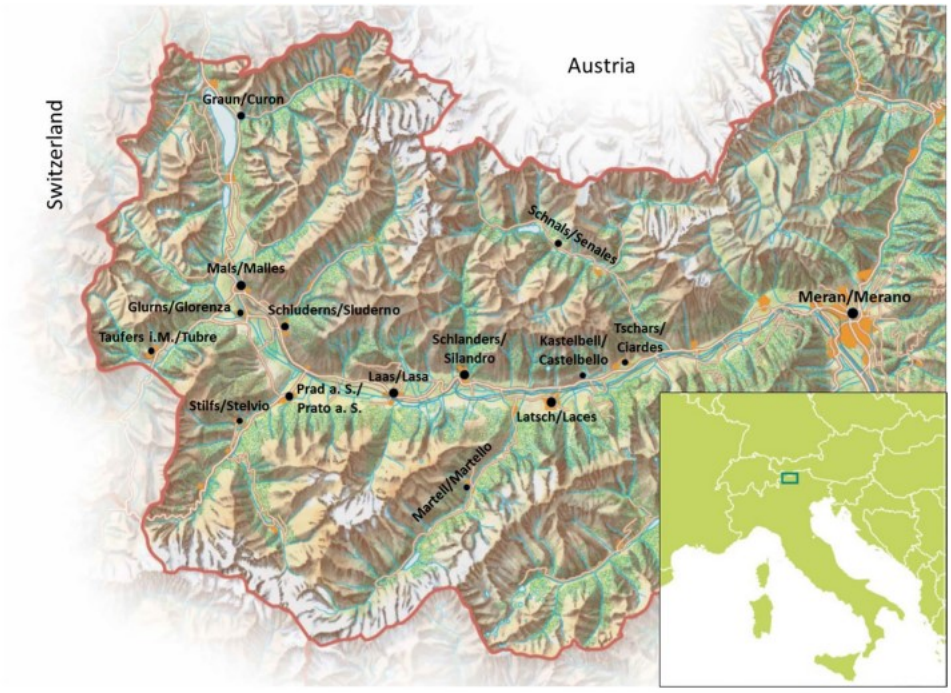


Figure 8: Geographical location of the CHRIS study. [49]

The target population in this restricted area of the Alps is characterized by a rather homogeneous life-style. Previous works demonstrated that the population in this area is stable, with low residential mobility across generations [50-52] and low inbreeding [53]. Such a homogeneity would constitute an advantage for the mapping of causal genetic variants in this geographical area [49].

All participants underwent blood drawing, urine collection, anthropometry analysis, and clinical assessments in the early morning following overnight fasting. Participants had been asked not to smoke or drink alcohol or caffeine-rich beverages

for at least 3 hours prior to the visit. Detailed medical history was reconstructed through both interviewer- and self-administered questionnaires. Participants were asked to report genealogical information about parents and grandparents, allowing the reconstruction of up-to-five generation pedigrees [54].

The CHRIS study is focused on cardiovascular, metabolic, neurological, and psychiatric health. The metabolic section referred to iron disorders of this project aimed to the definition of a reference range for serum hepcidin, ERFE and sTfR levels, as a function of age, gender, and physiologic status with diagnostic insights about iron metabolism disorders. A second aim was to partially replicate data emerged in the Val Borbera population described by Traglia et al. [55] evaluating the age- and sex-dependent variations of transferrin saturation, serum ferritin, serum hepcidin, hepcidin/ferritin ratio and ERFE in the whole population. Finally, this project aimed to investigate the correlation between hepcidin, ERFE, sTfR and hematological parameters (Hb, Ht, MCV, MCH, RBC count), iron status markers (transferrin saturation, serum iron, ferritin) and other biochemical data available in the CHRIS study dataset.

A genome-wide association study (GWAS) was also planned by our collaborators involved in this study, in order to reveal genetic variants associated with iron status and to discover potential new pathways involved in hepcidin, erythroferrone and soluble transferrin receptor regulation. All 4979 subjects involved in this part of the project have been genotyped and are available for GWAS.

Genome-Wide Association Study

The genome-wide association study (GWAS) detects common genetic variants that associate with complex genetic disorders (they are referred to as complex because their origin is not driven by one genetic factor, but rather by many genes and gene-environment interactions). The main biomarker under study is single nucleotide polymorphisms (SNPs) that usually consist of two alleles. The frequency of the rarer of the two alleles is often referred to as the minor allele frequency (MAF). In GWAS, the majority of SNPs are common variants with MAF greater than 5% [56, 57]. Thus, GWAS is based on the hypothesis that common genetic variants explain some of the observed phenotypic variance for complex traits [58]. The goal of

GWAS is the identification of trait-associated SNPs in order to reveal new insights into the biological mechanisms underlying these phenotypes and subsequently develop translational prevention or treatment strategies [59]. Also in field of iron disorders, GWAS in a broad population have discovered common genetic variants associated with iron metabolism that might confer a susceptibility to iron disorders [60, 61].

A typical GWAS workflow comprises 5 steps (Figure 9):

1. Genotyping: the initial phase of a typical GWAS consists in genotyping DNAs from a few hundred cases and few hundred controls by either arrays (Affimetrix or Illumina) and Next-Generation Sequencing (WES or WGS). Many genotypes are then analyzed in relation to disease in a case-control analysis, or in relation to quantitative trait phenotypes by regression analysis. This will then identify one or more genetic variants or SNPs to be associated with a disease or phenotype. Thus, a successful GWAS starts with having available a large collection of DNA samples of well-phenotyped subjects. This can be a case-control collection or subjects from a cohort study [62].
2. Quality Control: Quality control procedures are crucial to remove low quality samples or markers, and to reduce the spurious associations in later analysis. In terms of sample quality control is important to identify individuals with discordant sex information (by calculating the homozygosity rate on the X-chromosome), samples with low DNA concentration and quality (could result in a poor genotyping call rate), closely related or duplicated subjects (by applying association tests that assume independence among subjects) and outlier subjects from the target cohort (since individuals with distant ancestry have systematic allele frequency differences from the cohort under investigation). Instead, in marker quality control it is important to detect SNPs with high proportion of missing genotypes (implying that SNPs are difficult to genotype, leading to a high rate of genotyping errors), SNPs that depart from Hardy Weinberg Equilibrium (HWE) (deviation from HWE is used as a tool to flag up SNPs with large numbers of genotyping errors) and SNPs with low MAF (more

easily affected by genotyping error as the rarer allele only occurs in a few individuals) [57].

3. Imputation: statistical imputation is a special step that allows many more SNPs to be analyzed than those that are on the GWAS genotyping array. It is facilitated by the fact that the genotypes of not directly genotyped variants can be estimated by the haplotypes inferred from directly genotyped SNPs and the haplotypes observed from a fully sequenced reference panel [63, 64].
4. GWAS analysis: SNPs in thousands of samples are then subjected to a statistical evaluation of association of genotype with the phenotype (trait) of interest. Linear regression is generally used to perform an analysis on quantitative traits and logistic regression on dichotomous traits. Even though other methods are proposed for GWAS, these two are the most frequently used methods since they also allow to adjust for confounders. The results for a GWAS analysis are typically plotted as a Manhattan plot, which provides exact p-values per SNP on each chromosome. It is commonly accepted that a p-value smaller than $5x10^{-8}$ is considered genome-wide significant, corresponding to the Bonferroni correction [57, 65].
5. Post-GWAS analysis: the post-GWAS analysis consist in the replication and validation of the genotype-phenotype association, in order to control how the findings are genuine. Replication studies should be conducted in independent data sets, should involve the same phenotype and should be conducted in a similar population [57].

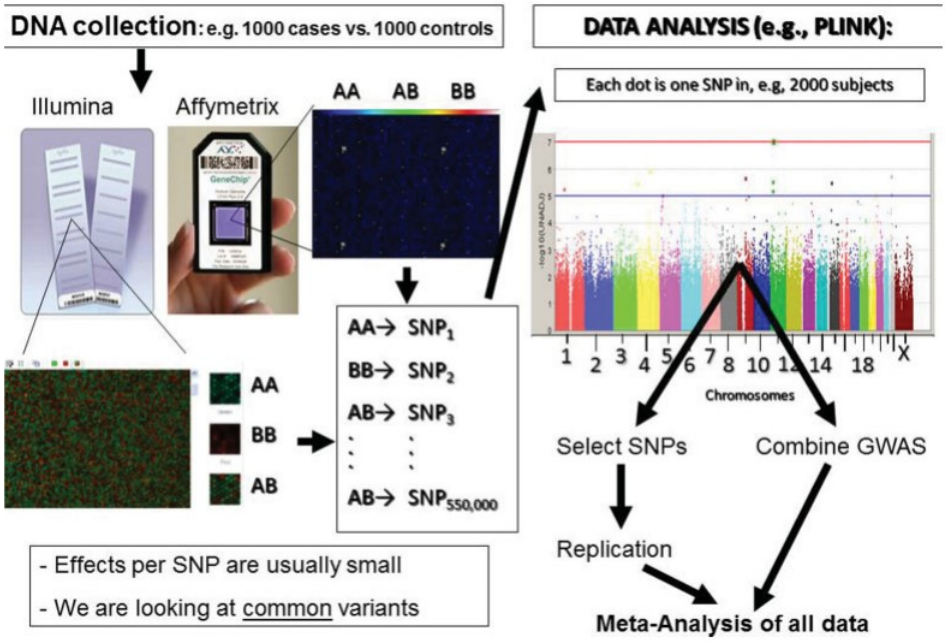


Figure 9: Schematic representation of the main steps of GWAS. [62]

AIM OF THE STUDY

The main aims of my PhD thesis project were:

- Evaluation of hepcidin (20, 24 and 25 isoforms), ERFE and sTfR in the homogeneous population of the middle and upper Vinschgau/Val Venosta by means of specific laboratory assays (LC-MS/MS approach for hepcidins and ELISA techniques for ERFE and sTfR).
- Exploration of the behavior of iron-related parameters and indicators in the whole CHRIS population by evaluating principally the age- and sex-dependent variations of transferrin saturation, serum ferritin, serum hepcidin, hepcidin/ferritin ratio, serum ERFE and serum sTfR and by obtaining reference ranges according to selected criteria.
- Correlation analysis between hepcidin, ERFE, sTfR and hematological parameters (Hb, Ht, MCV, MCH, RBC count), iron status markers (transferrin saturation, serum iron, ferritin) and other biochemical data available in the CHRIS study dataset.
- Identification of genetic determinants of serum hepcidin by GWAS in the CHRIS population.

MATERIAS AND METHODS

CHRIS Study Subjects

The Vinschgau/Val Venosta population has been previously described in detail in a descriptive paper [49]. Only individuals aged 18 years or older were eligible to participate in the study. The CHRIS study was approved by the Ethical Committee of the Healthcare System of the Autonomous Province of Bolzano (Südtiroler Sanitätsbetrieb/Azienda Sanitaria dell'Alto Adige, protocol no. 21/2011 (19 Apr 2011)). In addition, the study is compliant with current Italian and EU regulations and with the Helsinki Declaration. Privacy and security in data handling and sharing are strictly enforced and a public access code regulates how data and samples can be used [49].

Blood Tests

Fasting blood samples (49 ml) were collected at the study center for laboratory analysis and biobanking. Fractioned blood was aliquoted in small volumes and stored at -80˚C. Biomarkers analyzed include all main markers of iron metabolism (serum iron, ferritin, transferrin, transferrin saturation (TSAT) and total iron-binding capacity (TIBC)).

Genomic DNA extraction and Genotyping

Genomic DNA extraction was performed using an automated platform for nucleic acid isolation (Chemagic Magnetic Separation Module I, PerkinElmer Chemagen Technologie GmbH, Germany) using a dedicated method based on magnetic beads (Chemagic DNA Blood Kit special, PerkinElmer Chemagen Technologie GmbH, Germany). DNA was eluted in 300 µl and the absorbance was determined using the Take3 Trio Micro-Volume Plate (Epoch, Take3 Trio Micro-Volume Plate, BioTek Instruments, USA). Finally, DNA was stored at –20 °C [49].

All CHRIS samples included in this project were genotyped on ~1 million SNPs with the Illumina HumanOmniExpressExome Bead Chip, which includes ~250.000 exonic variants. Moreover, Whole Exome Sequencing (WES) was also performed for 3316 subjects.

Serum Hepcidin Assay

Hepcidin measurement was performed using an updated and validated Mass-Spectrometry (MS)-based assay. This analysis allowed the quantification of the mature bioactive circulating isoform (hepcidin-25) and two smaller isoforms (hepcidin-24 and hepcidin-20), using a chromatography-tandem mass spectrometry (LC-MS/MS) approach [66]. Hepcidin-25 synthetic standards (the native and the isotopic labelled internal standard), and standards for hepcidin-24 and hepcidin-20 isoforms, were purchased from Peptide International (Lousiville, USA).

Blank serum, deprived of hepcidin, was prepared using charcoal treatment. The calibration curve was prepared with the blank serum and a known concentration of standards of each hepcidin isoform. The curve was created in eight points with concentrations ranging from 100 nM to 0,5 nM.

Solutions needed for hepcidin quantification were prepared as follow (Table 2).

SOLUTION	REAGENTS
Internal Standard Solution (ISS)	33,8/66,2/0,1 (v/v/v) acetonitrile/H_2O/trifluoroacetic acid (TFAA)
Internal Standard Working Solution (ISWS)	979,59 µl of ISS + 20,41 µl of Hepc-25* (the isotopic labeled) to a final concentration of 400 nM
Formic Acid (FA) 0,1%	10 µl of FA in 10 ml of H_2O
Wash Solution (WS)	30/75/5 (v/v/v) methanol/H_2O/NH_3
Elution Solution (ES)	72,5/22,5/5 (v/v/v) acetonitrile/ H_2O/formic acid

Table 2: List of solutions and its respective reagents used in the hepcidin measurement.

Sample preparation was done in the same way for both calibration curve and patients' serum by adding 12 µl of ISWS and 40 µl of FA 0,1% to 80 µl of serum (or the points of calibration curve). Finally, 200 µl of H_2O was added after vortex and briefly spin.

Once the samples have been prepared the next step was the solid-phase extraction using Oasis® HLB µElution plate 30 µm (Waters, Italia). The elution plate was applied on the filtration system, its columns were conditioned with 200 µl of methanol followed by 200 µl of H_2O. The serum samples were transferred onto the columns that were washed with 200 µl of H_2O, 200 µl of WS and finally 200 µl of H_2O. The hepcidin peptides were then eluted twice with 25 µl of ES and finally 60 µl of H_2O was added. Samples were loaded into the LC-MS/MS.

The chromatographic separation was performed with 1290 LC (Liquid Chromatography) system (Agilent). 25 µl of sample were injected into the column that was an X-Terra MS C18 which allows the peptides separation based on the hydrophobicity. The analytes distributed between the polar mobile phase (MP) consisting in 0.1% FA in water (mobile phase A) and the hydrophobic (non-polar) stationary phase consisting in the C18 column. The trapped peptides were eluded using organic phase gradient (mobile phase B) consisting in 0.1% FA in 90/10 (v/v) acetonitrile/water.

The tandem mass spectrometry detection was performed using an Agilent Technologies 6460 Triple Quad LC-MS/MS. The acquisition was performed in multiple-reaction monitoring (MRM) mode. The precursor ion was selected in the first chamber, after its fragmentation in the collision chamber only the fragment with the specific mass over charge (m/z) values were selected to be analysed in the second chamber. For the identification of hepcidin-25 two parameters were used: transition and retention time. The transitions used were: 930.5→1145.0 m/z for quantification ion for hepcidin-25 and 930.5→353.7 m/z for qualification ion for hepcidin-25. This means that in the first quadrupole was selected the precursor ion that corresponded at a mass over charge ratio of 930.5, this was collided in the collision chamber and only product ions with m/z that corresponded to 1145.0 and 353.7 were analysed in the second quadrupole. The transition for the isotopic labelled hepcidin-25 was 937.7→1150.0 m/z. The second parameter was the retention time that for hepcidin-25 ranged from 3.16 to 3.5 seconds. The limit of detection (LOD) of hepcidin measurement using a liquid chromatography tandem mass spectrometry (LC-MS/MS) approach is 0.5.

Serum ERFE Assay

Erythroferrone analysis was performed using the Human Erythroferrone IE™ ELISA kit (Intrinsic Lifesciences-The BioIron Company™, USA), which is a double monoclonal antibody sandwich ELISA assay. The measurements were obtained according to manufacturer instructions. In brief, after the incubation of serum samples in the assay plate pre-coated with mouse anti-human erythroferrone monoclonal antibody, a second monoclonal anti-erythroferrone antibody conjugated to horseradish peroxidase was added. The quantification of the enzyme conjugate bound to the immobilized ERFE was done by the addition of substrate, then after stopping the reaction with an acid solution, the plate was read at 450 nm in a PowerWave™ XS Microplate Reader (BioTek Instruments). The standard curve was prepared with Human erythroferrone standard concentrations ranging from 10 ng/ml to 0.16 ng/ml (8 points). Samples, standards and controls were assessed in duplicates and diluted (1:10 dilution). Absorbance values were used for data interpolation and reduction by GraphPad Prism 8 software (San Diego, CA, USA). Concentrations were expressed in ng/ml. Samples presenting a CV > 14% in repeated measurements were re-assayed. The LOD of Human Erythroferrone IE™ ELISA kit is 0.16.

Serum sTfR Assay

Soluble Transferrin Receptor was measured using the DRG sTfR ELISA kit (DRG Instruments GmbH) and performed according to manufacturers' instructions. This ELISA assay is a sandwich ELISA, where the microtiter wells were coated with a monoclonal (mouse) antibody directed towards a unique antigenic site of the sTfR molecule. An aliquot of patients' serum containing endogenous sTfR were incubated in the coated well with enzyme conjugate, which is an anti-sTfR antibody conjugated with horseradish peroxidase. After adding the substrate solution, the intensity of color developed was proportional to the concentration of the sTfR in patients' samples. The standard curve was prepared in six points with sTfR standard concentrations ranging from 160 ng/ml to 10 ng/ml. The concentration of sTfR was obtained from the mean absorbance of the standard curve. Samples, standards and controls were assessed in duplicates and diluted (1:51 dilution). Absorbance values were used for data interpolation and reduction by GraphPad Prism 8 software (San

Diego, CA, USA). Concentrations were expressed in ng/ml. The normal expected values reported by DRG sTfR ELISA's datasheet is 480-3090 ng/ml (min.-max.) and a mean of 1430 ng/ml, observed in a group of 176 healthy human subjects. The absorbance was read at 450 nm on the PowerWave^{TM} XS Microplate Reader (BioTek Instruments). Samples presenting a CV > 14% in repeated measurements were re-assayed.

Statistical Analysis

All calculations were performed using Python software version 3.9.1 and GraphPad prism 9 (GraphPad software, San Diego, CA) statistical packages. Data distribution analysis and normality test were performed for all numeric variables. The normality test was based on D'Agostino's K-squared test, that aims to establish whether or not the given sample comes from a normally distributed population.

Comparisons of all measured parameters in men and women were performed using the Mann-Whitney U test, a non-parametric test of the null hypothesis that, for randomly selected values X and Y from two populations, the probability of X being greater than Y is equal to the probability of Y being greater than X. Quantitative data were also analyzed by ANOVA with polynomial contrast for linear trend when indicated. Sex specific correlations between quantitative variables were assessed using Spearman's test, specially to measure the extent to which hepcidin (25 and 24 isoforms) and other parameters are related. After that, simple and multiple linear regression analyses were performed to find the best independent predictors of serum hepcidin.

In subjects with undetectable hepcidin, ALT, CRP and ERFE, the arbitrary numeric values of 0.1, 1, 0.001 and 0.16 were added respectively. Specially for hepcidin and ERFE, which had a significant number of undetectable subjects, the arbitrary value was added for a correct log-transformation, when appropriate.

Two-tail P values < 0.05 were considered as statistically significant.

It is important to note that ERFE statistical analyses were performed in collaboration with the EURAC Center of Research (Bolzano, Italy). The statistical

analysis of ERFE and sTfR data is still ongoing, therefore it was possible to report in this manuscript only preliminary results.

Genetic determinants investigation

Genome-wide association analysis of hepcidin-25 was performed in CHRIS participants, in order to study the association of SNVs with serum hepcidin in this population. Genetic association analysis and single variant association analysis were performed using the HRC (Haplotype Reference Consortium) imputed genotype data, the TopMed (Trans-Omics for Precision Medicine) imputed genotype data and the whole exome sequencing data. These three different approaches were used together because they are complementary, therefore each one contains different, only partially overlapping, sets of variants. Single variant association analysis was performed using EMMAX (Efficient Mixed-Model Association eXpedited) tool and was run separately.

RESULTS AND DESCUSSION

CHRIS POPULATION DISTRIBUTION

The metabolic section referred to iron disorders of this project analyzed anthropometric data and iron status parameters in 4979 subjects of the CHRIS population, consisting of 2212 males and 2767 females (age range 18-93 and mean age 46.2±16.4 years). The age and sex distribution of this population is reported in Figure 10.

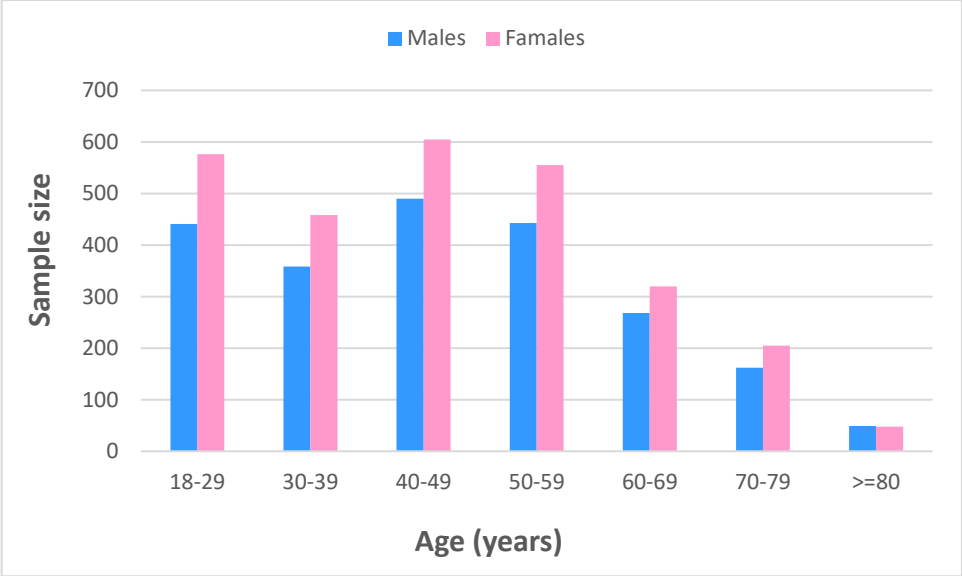


Figure 10: Age and sex distribution of the CHRIS individuals. In blue are indicated males, in pink are shown females subjects.

DATA VISUALIZATION AND VARIABLES DISTRIBUTION

Anthropometric data, red cell parameters and different iron status biomarkers were available for all samples (Table 3). Figure 11 shows the histogram with the distribution of the data. As we can see from the graphs, some distributions are right skewed, in particular ferritin, CRP, ALT, triglyceride, hepcidin and hepcidin/ferritin ratio, so they do not seem to follow a normal distribution. Moreover, according to the normality test, not all variables resulted to be normal with p-value > 0.05, so we decided to use a non-parametric test to compare the distributions between males and females.

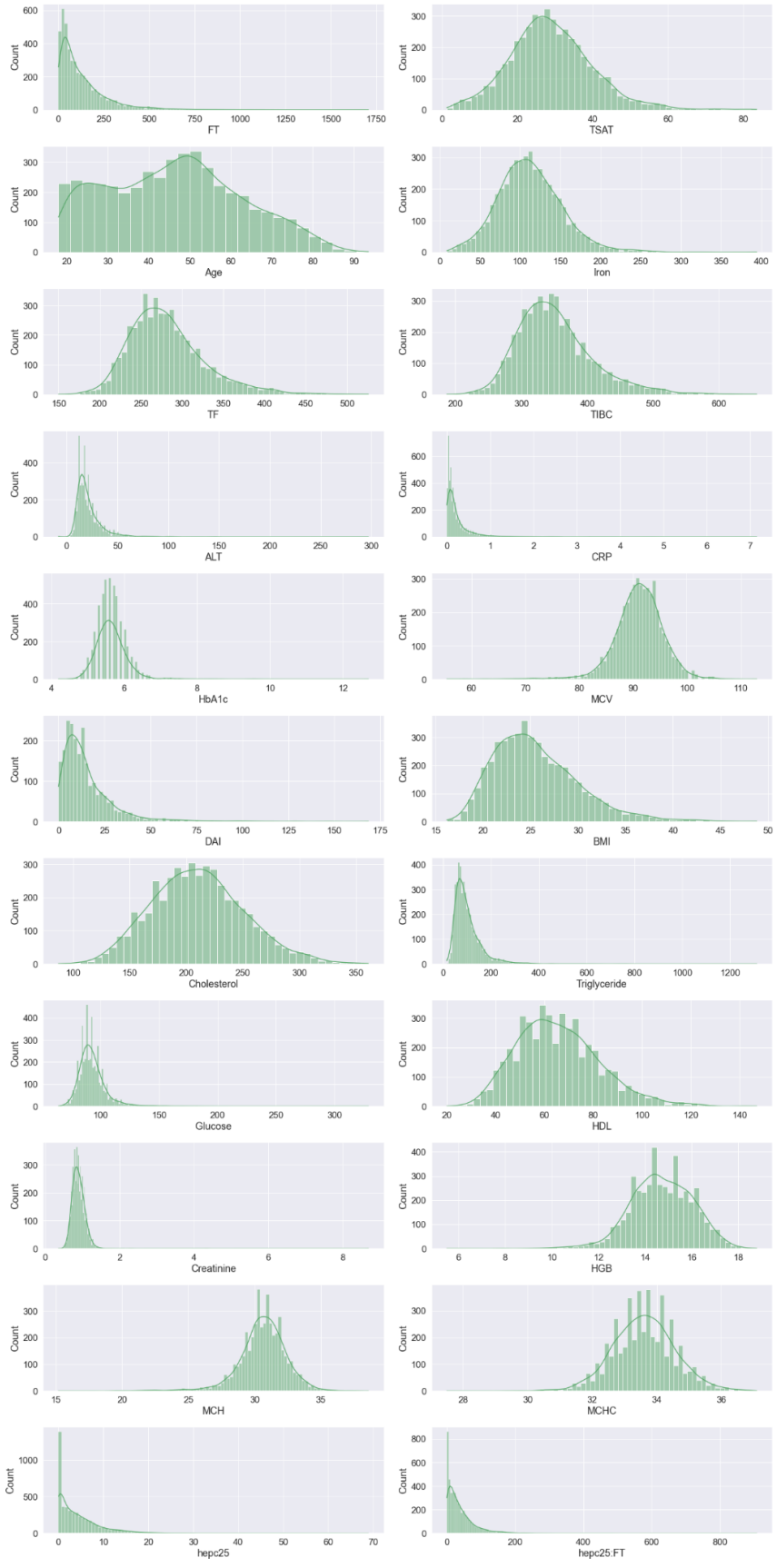


Figure 11: Histograms showing the distribution of each variable.

Mann-Whitney U test was performed to check differences in the distribution between males and females (Table 3). The variables Age, HbA1c, MCV and ERFE had the same distribution between males and females. Age, HbA1c and MCV histograms were displayed for visual confirmation (Figure 12).

Trait	Units	Males			Females			p°
		N	Median	CI	N	Median	CI	
Age	years	2212	46.6	45.92-47.3	2767	45.94	45.33-46.54	NS
BMI	Kg/m2	2162	25.8	25.63-25.96	2723	23.9	23.7-24.08	****
HGB	g/dl	2211	15.7	15.65-15.74	2761	14	13.96-14.03	****
MCV	Fl	2211	91.2	91.01-91.38	2761	91.3	91.12-91.47	NS
MCH	pg	2211	30.8	30.73-30.86	2761	30.6	30.52-30.67	****
MCHC	g/dl	2211	33.8	33.76-33.83	2761	33.4	33.36-33.43	****
Iron	µg/dl	2210	114	112.38-115.61	2765	106	104.5-107.49	****
TIBC	µg/dl	2212	333	331.14-334.85	2767	353	350.58-355.41	****
Ferritin	ng/ml	2212	163.9	157.23-170.66	2767	45.4	42.74-48.05	****
Transferrin	mg/dl	2212	267	265.51-268.48	2767	283	281.06-284.93	****
TSAT	%	2210	30.3	29.85-30.74	2765	26.3	25.89-26.7	****
Hepcidin-25	nM	2073	4.58	4.33-4.82	2571	1.42	1.26-1.57	****
Hepcidin/Ferritin	pmol/µg	2073	24.69	23.33-26.05	2571	27.09	24.78-29.4	**
ERFE •	ng/ml	1680	0.69	0.25-1.61	2047	0.65	0.2-1.77	NS
Cholesterol	mg/dl	2212	207.5	205.76-209.23	2767	210	208.49-211.5	****
HDL cholesterol	mg/dl	2212	57	56.39-57.6	2766	71	70.38-71.61	****
Triglycerides	mg/dl	2212	93	89.82-96.17	2767	82	80.11-83.88	****
HbA1c	%	2209	5.6	5.57-5.62	2763	5.6	5.58-5.61	NS
CRP	mg/dl	2205	0.11	0.09-0.12	2762	0.14	0.12-0.15	****
Creatinine	mg/dl	2212	0.99	0.981-0.998	2767	0.8	0.79-0.8	****
ALT	U/l	2212	23	22.32-23.67	2767	15	14.66-15.33	****
Daily alcohol intake (DAI)	g	1461	13.91	13-14.81	993	8.41	7.83-8.98	****
Glucose	mg/dl	2212	94	93.36-94.63	2767	88	87.54-88.45	****

Table 3: Characteristics of the CHRIS population according to sex.
***p<0.01, ****p<0.0005.*
BMI: Body Mass Index; CRP: C-Reactive Protein; HGB: hemoglobin; HbA1c: glycated hemoglobin; MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; TIBC: total iron biding capacity; TSAT: transferrin saturation; ALT: alanine aminotransferase; MCHC: mean corpuscular hemoglobin concentration; N: Sample size; CI: 95% confidence interval; NS: not significant. • ERFE values are represented by median and interquartile range (IQR).

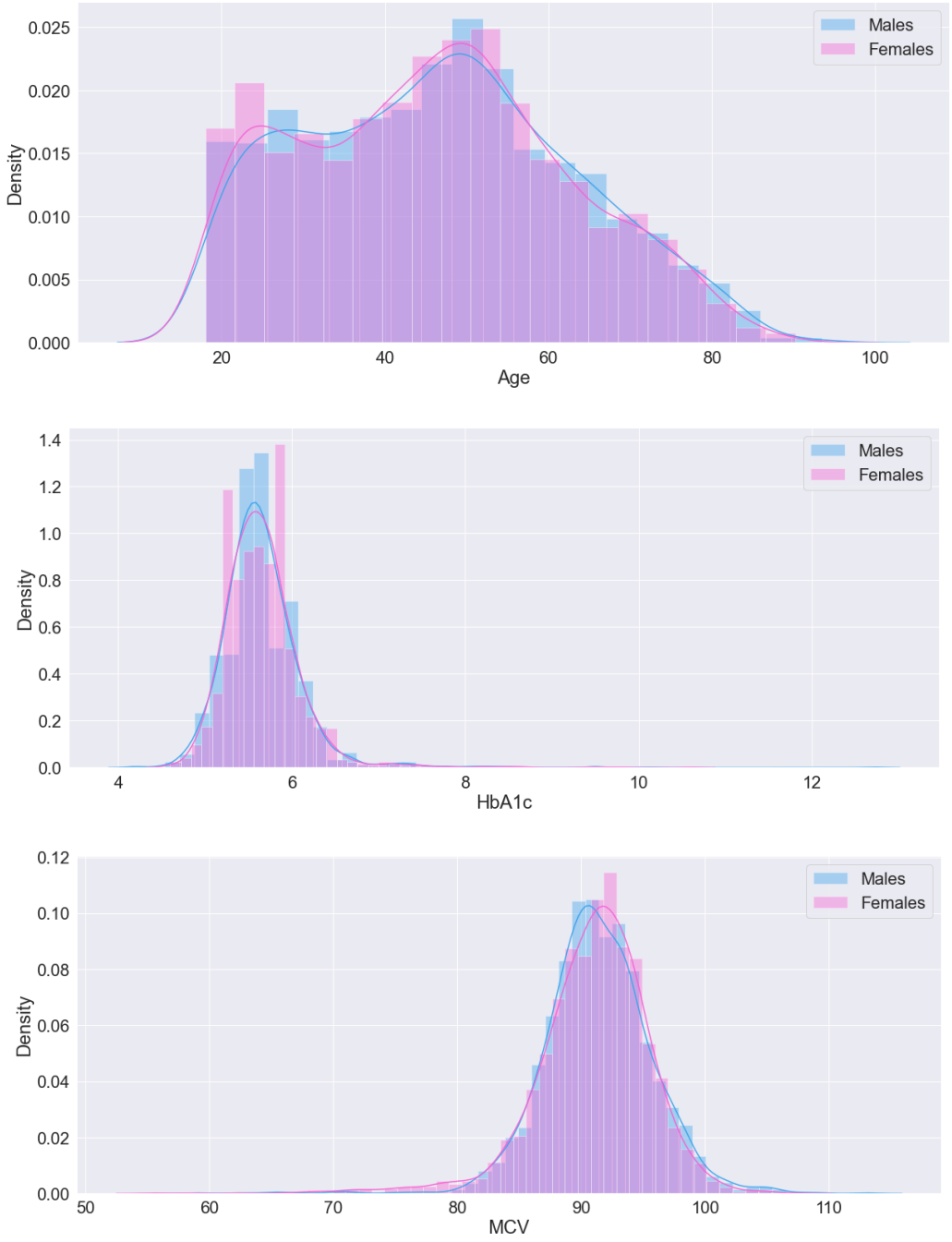


Figure 12: Histograms showing the distributions of Age, HbA1c and MCV in males and females, respectively.

HEPCIDIN-25 ANALYSIS IN THE CHRIS POPULATION

Serum hepcidin was measured by a mass spectrometry (LC-MS/MS) approach in 4644 serum samples of the CHRIS population (2073 males and 2571 females; age range 18-93 and mean age 46.23±16.41 years). Hepcidin-25 distribution according to age and sex are reported in Table 4.

Age Class	N	Males	N	Females
18-29	415	3.62 (3.07-3.96)	538	0.1 (0.1-0.6)
30-39	336	4.43 (3.84-5.41)	418	0.7 (0.1-0.94)
40-49	455	5.14 (4.76-5.55)	563	0.1 (0.1-0.67)
50-59	409	4.74 (4.2-5.49)	523	2.55 (1.94-3.0)
60-69	257	5.25 (4.58-5.92)	295	4.76 (3.81-5.43)
70-79	154	4.64 (3.99-5.39)	193	3.65 (2.78-4.21)
>=80	46	4.46 (2.81-6.49)	41	3.59 (2.14-4.7)

Table 4: Serum hepcidin-25 levels by age and sex. Values are represented as median (nM) with 95% of CI. N: sample size.

Figure 13 shows the variation of hepcidin, ferritin, transferrin saturation, and hepcidin/ferritin ratio according to sex and age in the whole population. Consistently with the results of a previous population study [55], hepcidin and ferritin showed robust age and sex dependent variations (Figure 13A and Table 4; Figure 13B), and the same was observed, even if in a lesser extent, for transferrin saturation (Figure 13C).

In males, serum hepcidin-25 levels were quite stable across the different age groups, while in females the distribution is characterized by more dynamic changes. Indeed, women before onset of menopause had hepcidin levels lower (1-2 nM) than in men of the same age (4-6 nM). The levels of hepcidin were more similar in males and females aged 60-69 (around 4-5 nM), while among the elderly, a significant decrease occurred in both genders, more clearly in women (Figure 13A).

Ferritin levels showed a trend similar to hepcidin, even though a more variable levels of ferritin were observed in men across all age groups. In women the ferritin levels demonstrated a strict variation depending on three main age groups. Women aged 18-49 years had the lowest values, which increased in the 50-70 years interval, and then tend to remain at the highest values after the seventh decade (Figure 13B).

The hepcidin/ferritin ratio, used to correct for hepcidin changes according to iron stores [67, 68], was also considered. This analysis could be properly done only in subjects with both hepcidin and ferritin detectable values, so undetectable hepcidin-25 subjects were excluded. The trend represented in Figure 13D clearly remarks a large difference between young males and females, that sharply decreases with ageing. We can observe that it is higher in females, even in age groups with hepcidin levels near to males, indicating that the threshold for hepcidin increase in response to body iron could be lower in females or that females could have a greater capacity than males to modulate hepcidin as a function of iron stores. These results are consistent with those reported by Traglia in the Val Borbera population [55].

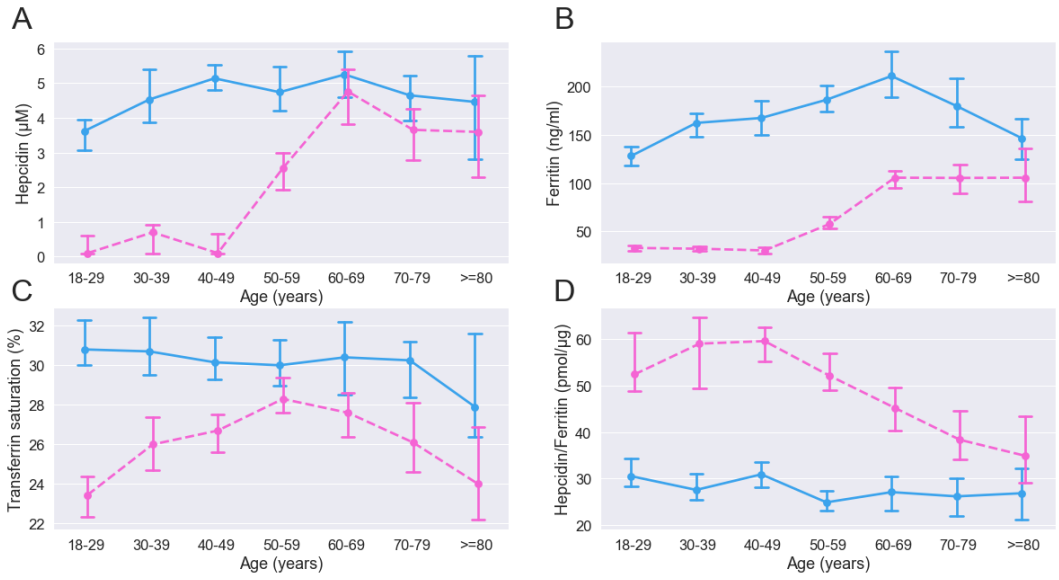


Figure 13: Age and sex dependent variations of serum hepcidin-25 (A), ferritin (B), transferrin saturation (C), and hepcidin/ferritin ratio (D) in the CHRIS population. Male are indicated by continuous blue line, females by a dotted pink line. Values are indicated as median with 95% CI.

Figure 14 showed the distribution of subjects with undetectable hepcidin-25 (N=1215) according to sex and different age groups compared to the entire population. As we can see, the number of individuals with undetectable hepcidin is mostly represented by premenopausal women (18-49 years).

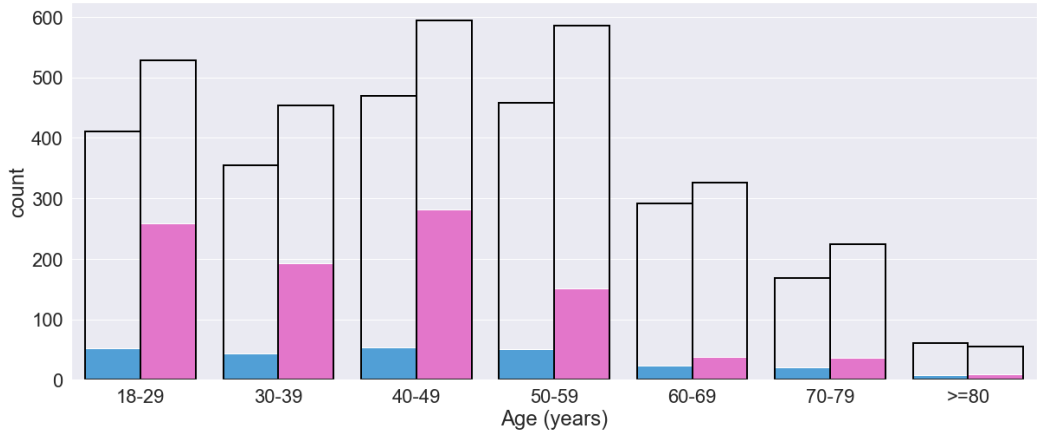


Figure 14: Age and sex distribution of undetectable hepcidin-25. Pink color indicates females while blue indicates males.

We reported the log-transformed hepcidin and log-transformed ferritin graphs in order to explain the number of undetectable hepcidin in the CHRIS population (Figure 15). The first peak of the log-hepcidin gragh (Figure 15 upper panel) rappresents the number of subjects with undetectable hepcidin, while the first peak of log-ferritin graph (Figure 15 lower panel) shows the number of subjects (males and females) having ferritin levels lower than 30 ng/ml. In order to better investigate the relation between undetectable hepcidin-25 and iron deficiency, we illustrated the distribution of undetectable hepcidin-25 subjects by dividing the values into three different classes of ferritin levels (Figure 16). As ferritin is an important biomarker for iron status, it is resonable that the high amount of subjects with undetectable hepcidin in our population could be explained, at least in part, by the high amount of subjects with low ferritin levels, especially premenopausal women, known to have highly prevalent low iron status [69]. However, a total of 531 subjects (181 males and 350 females) with hepcidin-25 undetectable were not explained by iron deficiency, thus remaining unexplained. Unexplained cases of undetectable hepcidin-25 could also depend on some analytical and pre-analytical caveats; some serum samples in fact could be rich in lipids, presenting high grade of turbidity and viscosity, thus leading to drawbacks in the sample preparation phase (SPE extraction) for MS analysis. Moreover, scarcity in the volume available for samples assessments required a scale down of the procedure and could have contribute to some performance reduction.

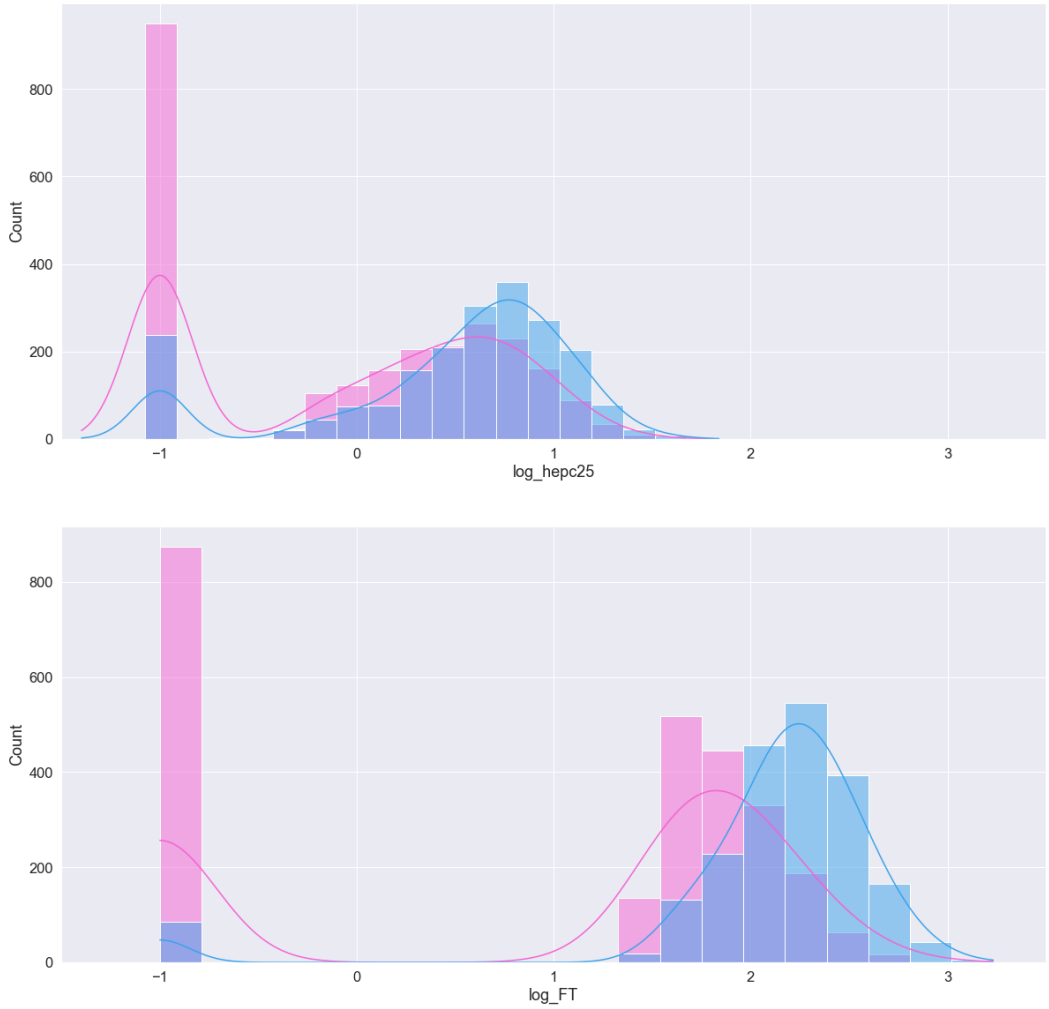


Figure 15: Log-hepcidin distribution (in the top panel) and log-ferritin distribution (in the bottom) by sex. Pink color indicates females while blue indicates males.

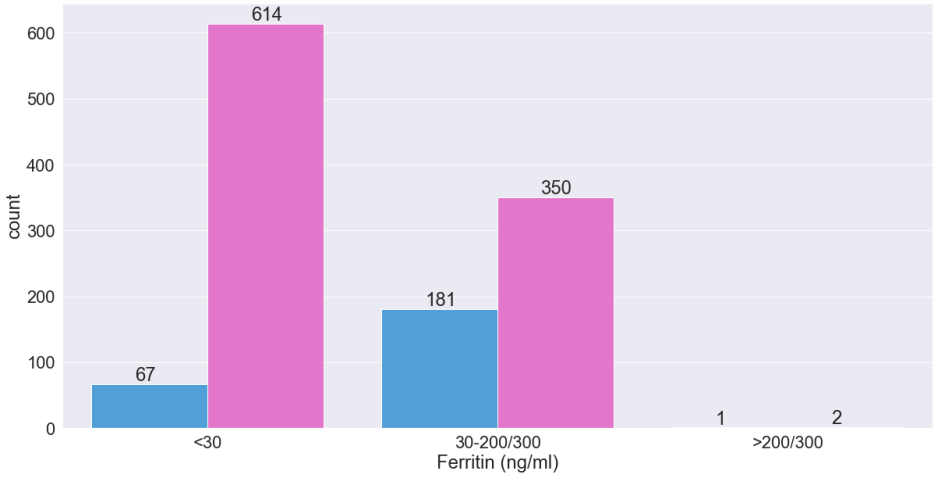


Figure 16: hepcidin-25 undetectable distribution into different ferritin levels. Pink color indicates females while blue indicates males.

Hepcidin-25 correlation analysis

Serum hepcidin correlation analysis by sex was performed using Spearman correlation. Figure 17 shows the r_s coefficient (Spearman's r) obtained in the correlation analysis of all variables, separated by males and females, for the whole population.

Serum hepcidin-25 correlation analysis by sex (Table 5) showed that Spearman's correlations were negligible ($|r_s| < 0.2$) for many variables (e.g. ALT, CRP, HbA1c, MCV, cholesterol, triglyceride, HDL and MCH), while were significant for ferritin ($r_s = 0.6$ males and 0.68 females) and transferrin saturation ($r_s = 0.23$ male and 0.32 female). Additional parameters had a significant Spearman correlation only in females, as iron ($r_s = 0.18$ males and 0.2 female), age ($r_s = 0.098$ males and 0.38 females) and transferrin ($r_s = -0.16$ males and -0.39 females). These results taken together confirm the known influence of iron status on hepcidin levels. Figure 18 summarizes all Spearman scores of hepcidin correlations with all variables; it is worth noting that correlations were almost always greater in females.

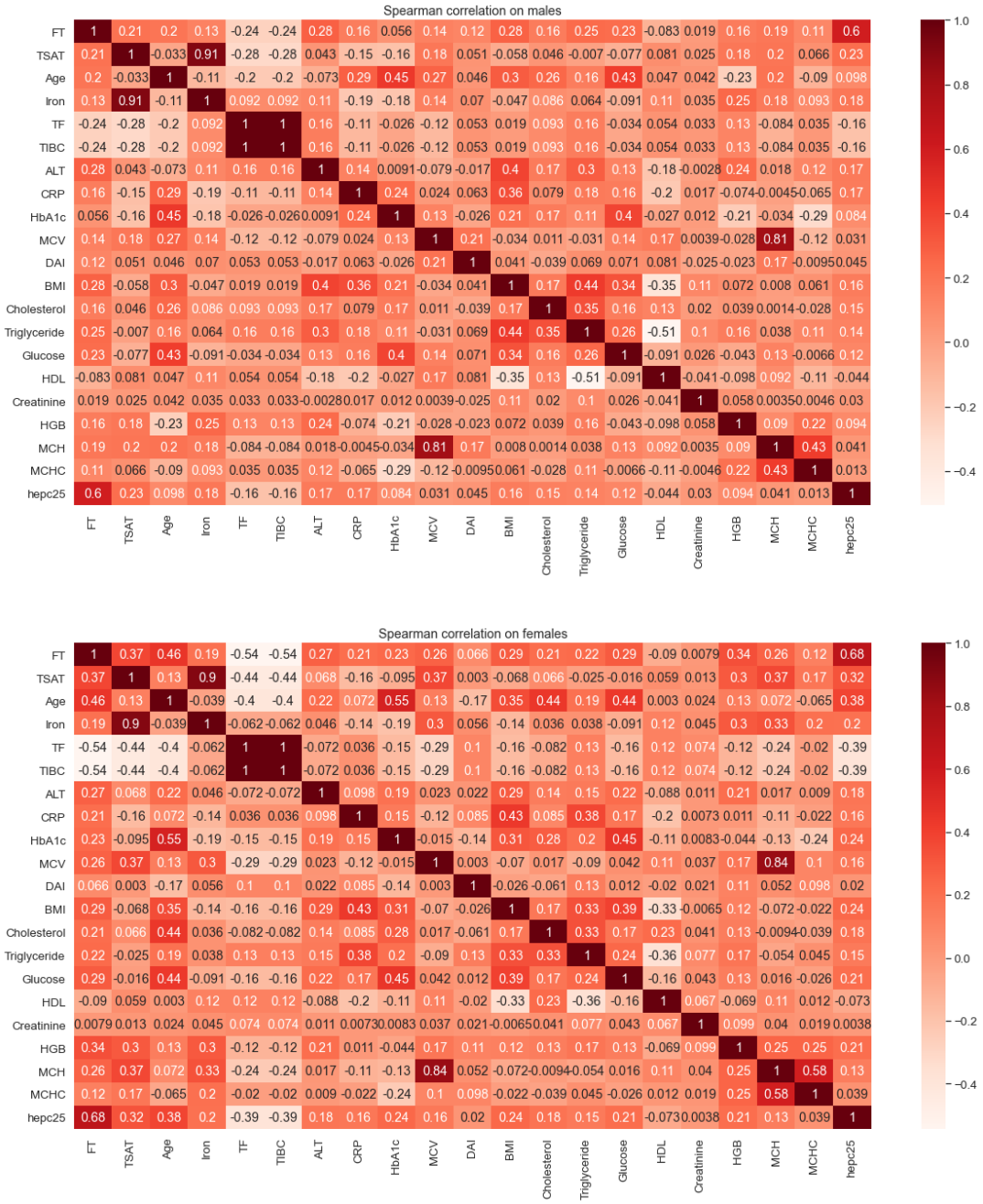


Figure 17: Spearman correlation with standard variables in males (upper panel) and females (lower panel). Correlation strength of $|r_s|$ coefficient: 1-0.8 very strong; 0.79-0.6 strong; 0.59-04 moderate; 0.39-0.2 weak; 0.19-0 very weak.

Traits	Males	Females
Ferritin	0.599*	0.68*
TSAT	0.226*	0.323*
Age	0.098*	0.379*
Iron	0.182*	0.196*
Transferrin	-0.16*	-0.38*
TIBC	-0.16*	-0.38*
ALT	0.174*	0.176*
CRP	0.169*	0.162*
HbA1c	0.083*	0.239*
MCV	0.03	0.159*
Daily alcohol intake	0.044	0.019
BMI	0.162*	0.237*
Cholesterol	0.147*	0.183*
Triglyceride	0.135*	0.152*
HDL	-0.04*	-0.07*
Creatinine	0.03	0.003
HGB	0.094*	0.209*
MCH	0.04	0.134*
MCHC	0.013	0.039*
Glucose	0.116*	0.207*

*Table 5: Sex specific correlation analysis of serum hepcidin-25. *Spearman's r with p-value < 0.05.*

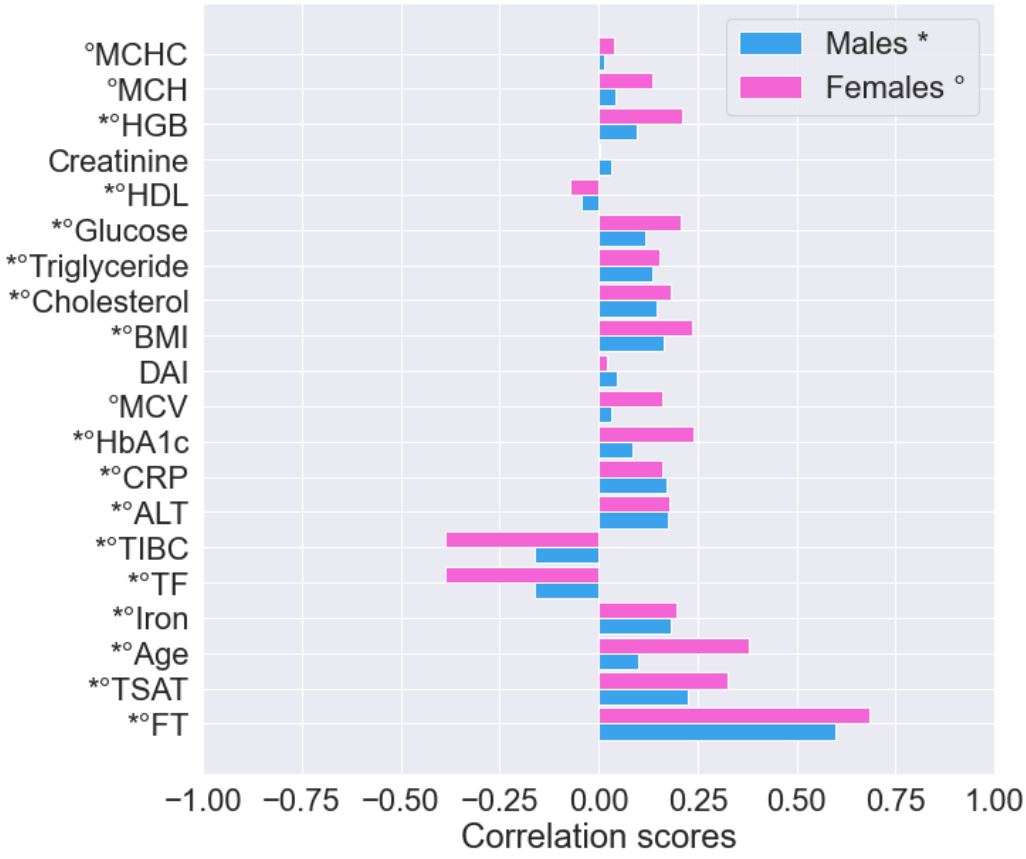


*Figure 18: Spearman correlation score of hepcidin-25 by gender. *statistically significant in males; °statistically significant in females.*

To study the associations between hepcidin-25 and other parameters, simple linear regression analyses were performed separately in males and females, using age as covariate. Before proceeding with simple linear regression, it was necessary to scale the data, since interpretation of regression coefficients is sensitive to the scale of the inputs. The log-transformation of hepcidin and others undoubtedly right skewed variables (ferritin, CRP, triglyceride and ALT) was also necessary. The choice of logarithmic transformation was justified by the fact that preliminary analysis showed an improvement in the regression models, in terms of normality of residuals (the difference between the observed value and the estimated value with linear regression model). Figure 19 shows quantiles of residuals' distribution of simple linear regression between hepcidin-25 and ferritin (upper panel) and quantiles of residuals' distribution of simple linear regression between log-hepcidin-25 and log-ferritin (lower panel) that overlaps the red line of normal distribution.

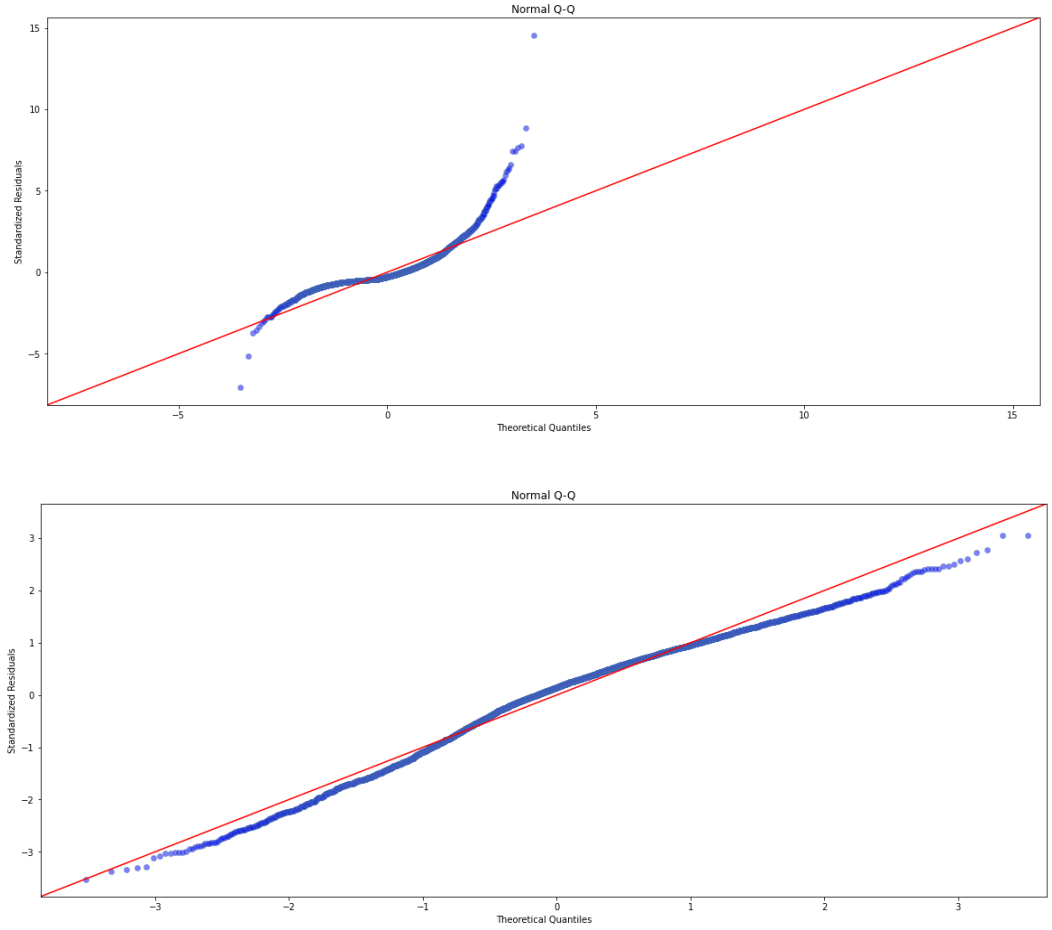


Figure 19: Quantiles of residuals' distribution of simple linear regression between hepcidin-25 and ferritin (on the top) and quantiles of residuals' distribution of simple linear regression between log-hepcidin-25 and log-ferritin (on the bottom).

Independent variables with significant linear relationship with hepcidin were tested in multiple linear regression models (Table 6). Ferritin, iron, ALT, CRP, age and cholesterol resulted independent predictors of hepcidin levels in males accounting for 41.6% of the total hepcidin variability; in females ferritin, iron, MCV and age were the independent predictors, accounting for 44.6% of its variability. Only ferritin, serum iron and age were common for both genders.

Trait	Males		Females	
	β	p	β	p
Log Ferritin	0.4013	****	0.4749	****
Iron	0.0639	****	0.0696	****
TF	-	-	-0.0237	NS
MCV	-	-	-0.0694	****
Log ALT	-0.0313	*	-	-
Log CRP	0.0701	****	-	-
Age	-0.0366	**	0.0767	****
BMI	-0.0125	NS	0.0152	NS
Cholesterol	0.0373	**	-	-
Log Triglyceride	-0.0241	NS	-	-
HGB	-0.0005	NS	-	-

*Table 6: Multiple regression analysis of hepcidin-25 by sex. * p<0.05, ** p<0.01, **** p<0.0005.*

Hepcidin-25 analysis in categorical parameters

Iron status:

The population (2073 males and 2571 females) was stratified in three classes according to ferritin levels, corresponding to iron deficiency (FT<30 ng/ml), normal iron balance (30≤FT≤200 ng/ml in females and 30≤FT≤300 ng/ml in males) and iron overload (FT>200 ng/ml in females and FT>300 ng/ml in males). Median hepcidin concentrations increased progressively with ferritin increasing and differed significantly among the three groups in males and females ($p<0.001$ in both genders); these results remained statistically significant also after adjusting for age and CRP (Figure 20). Similar results were obtained considering classes of transferrin saturation that define iron deficiency (TSAT<16%), normal iron status (16%≤TSAT≤45%) and iron overload (TSAT>45%). Hepcidin levels raised progressively among the three groups, with transferrin saturation increasing significantly in males and females ($p<0.001$ in both genders); such results remained statistically significant also after adjusting for age, ferritin and CRP (Figure 21).

The trends showed in Figure 20 and Figure 21 confirm the result of serum ferritin as a predictor of hepcidin concentrations and reflects the response of hepcidin to iron stores (represented by ferritin) and circulating iron (represented by serum iron and transferrin saturation) in both genders. These results are only partially

consistent with those reported in Val Borbera study, where no significant variations in hepcidin levels were observed in the three different classes of transferrin saturation [55].

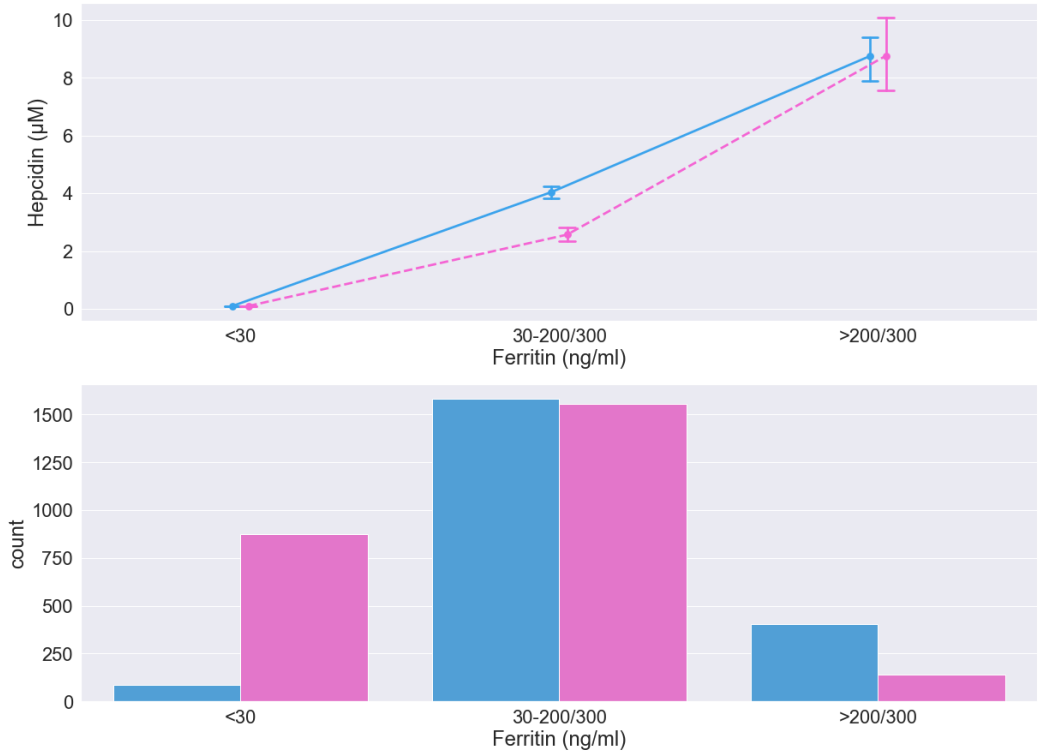


Figure 20: hepcidin-25 levels in groups of individuals classified according to their serum ferritin levels. Three classes are shown: iron deficiency (FT<30 ng/ml), normal iron balance (30≤FT≤200 ng/ml in females and 30≤FT≤300 ng/ml in males) and iron overload (FT>200 ng/ml in females and FT>300 ng/ml in males) (top panel). Number of males and females in each class (bottom panel). Males are indicated in blue, females in pink.

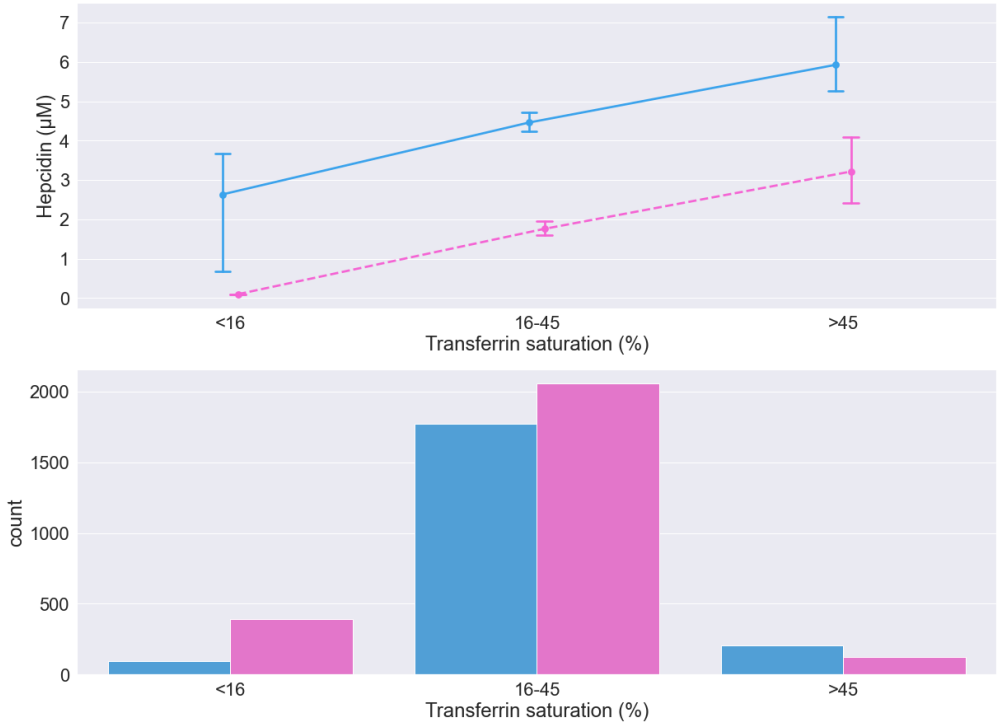


Figure 21: hepcidin-25 levels in groups of individuals classified according to their transferrin saturation levels. Three classes are shown: iron (TSAT<16%), normal iron status (16%≤TSAT≤45%) and iron overload (TSAT>45%) (upper panel). Number of males and females in each class (lower panel). Males are indicated in blue, females in pink.

Type 2 diabetes mellitus:

There are different studies highlighting the role of hepcidin in the pathogenesis of type 2 diabetes mellitus (T2DM), since iron overload was frequently linked and observed in patients affected by this condition [70]. As mentioned before (*Iron Metabolism and Hepcidin* section), hepcidin deficiency (typical condition of hemochromatosis) results in the development of iron overload and iron accumulation in different organs. Moderately increased iron levels, lower than those found in hemochromatosis, contribute to the development of T2DM by causing oxidative stress injury in hepatocytes and pancreatic β cells [71]. Moreover, hepcidin levels were reported to be lower in patients with T2DM compared to control subjects [72]. Besides low hepcidin, high hepcidin was also found in iron-related T2DM and dysmetabolic iron overload syndrome. A previous study indicated that hepcidin tends to progressively increase in response to a moderate increase of iron stores, remaining to be addressed the *primum movens* that determines the increasing of iron stores in these conditions [73]. To better

investigate the variation of hepcidin concentration in T2DM condition, a total of 210 subjects of CHRIS population (96 males and 114 females) were classified as having T2DM (Figure 22) in order to be compared with a control group. Differences in hepcidin concentration in subjects affected by T2DM not remained statistically significant after adjusting for age, ferritin and CRP, known confounders of hepcidin levels (Figure 22). These results are consistent with a recent meta-analysis study, where hepcidin was not found to be associated with the risk of T2DM [74].

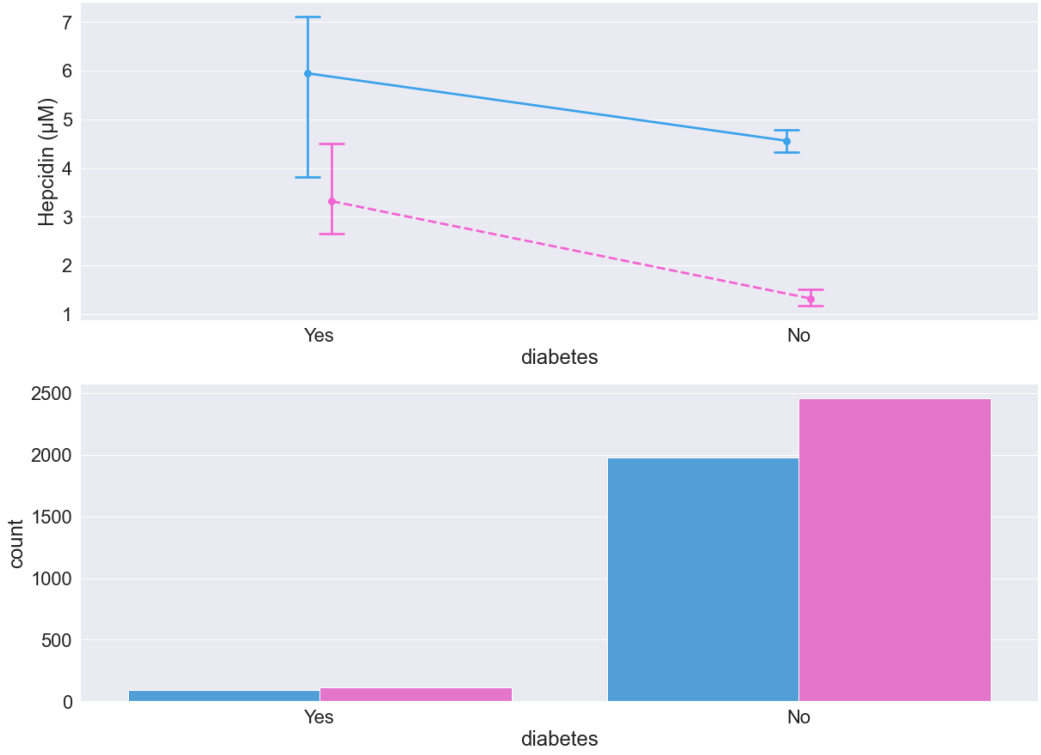


Figure 22: Variation of hepcidin concentration in a group of T2DM subjects compared to a non-T2DM group. Males are indicated by a continuous blue line, females by a dotted pink line. Males unadjusted p=0.38 (adjusted p=0.42); females unadjusted p<0.001 (adjusted p=0.35).

Metabolic syndrome:

The metabolic syndrome (MetS) refers to the co-occurrence of several known cardiovascular risk factors (including insulin resistance, obesity, atherogenic dyslipidemia and hypertension) and also hepatic diseases (nonalcoholic fatty liver disease) [75]. In this project we selected a subgroup of CHRIS population individuals with available complete data allowing their classification according to established criteria for metabolic syndrome [76]. In detail, the following features were considered:

1) Presence of waist circumference ≥ 94 cm in men and ≥ 80 cm in women.
2) Presence of diabetes mellitus or drug treatment for elevated blood glucose.
3) Triglyceride > 150 mg/dl.
4) HDL cholesterol < 40 mg/dl in men and < 50 mg/dl in women.
5) Blood pressure ≥ 130/85 mmHg or drug treatment for elevated blood pressure.

A total of 246 subjects that had at least three of the five traits mentioned above were considered to have MetS and were included in this analysis. None of the selected subjects were homozygotes for C282Y or compound heterozygotes for C282Y/H63D on *HFE* gene. As reported in Figure 23, MetS subjects seemed to have higher serum hepcidin if compared with subjects without MetS. In women, these results remained statistically significant after adjusting for age, ferritin and CRP ($p<0.001$). These results are only partially consistent with the ones reported by Martinelli and colleagues, where hepcidin tended to progressively increase with increasing numbers of the five MetS features in men and women. However, their multiple logistic models showed that MetS was independently associated with hepcidin in females but not in males [73]. These results suggest that the link between iron and dysmetabolic features may be particularly relevant in women [77, 78].

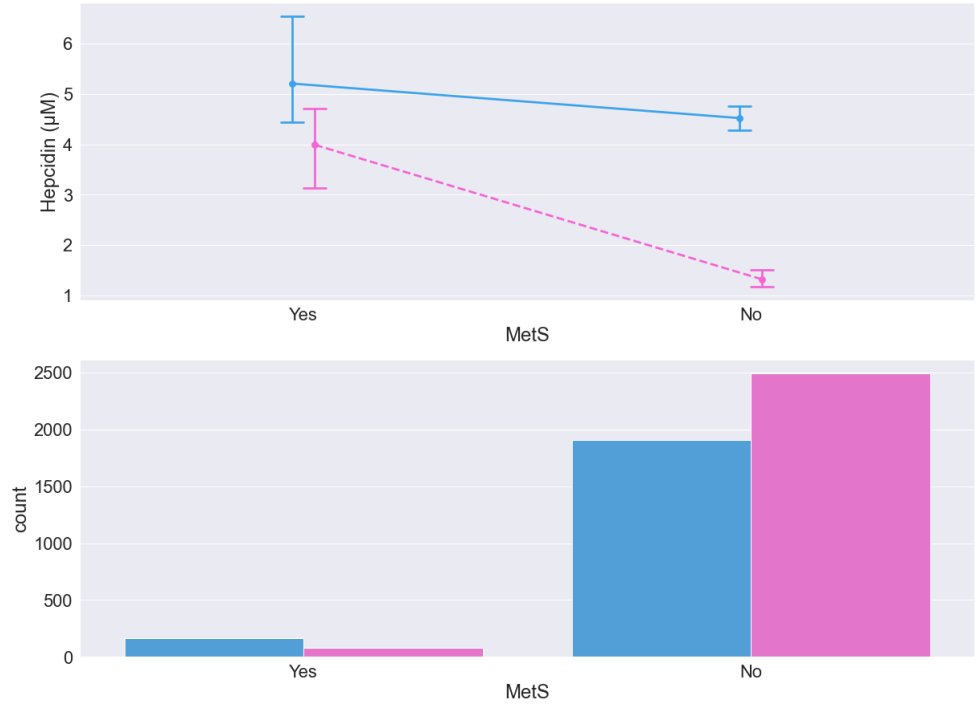


Figure 23: Variation of hepcidin concentration in subjects with and without MetS. Males are indicated by a continuous blue line, females by a dotted pink line. Males unadjusted p=0.01 (adjusted p=0.2); females unadjusted and adjusted p<0.001.

Dyslipidemia:

Dyslipidemia is a condition in which the amount of lipids is abnormal. It is an important component of MetS and a well-established risk factor for cardiovascular diseases (CVDs). Moreover, it is associated with obesity, hypertension, T2DM and other morbidities [79]. In order to investigate differences in hepcidin levels between dyslipidemic and non-dyslipidemic subjects, a group of 3129 individuals was selected from the CHRIS population that had at least one of the following features:

- Total cholesterol > 200 mg/dl.
- Triglyceride > 150 mg/dl.
- HDL cholesterol < 40 mg/dl in men and < 50 mg/dl in women.

Dyslipidemic subjects represents the 67.3% of the whole population (Figure 24, lower panel). Moreover, dyslipidemic males and females showed a statistically significant increment in hepcidin levels when compared with non-dyslipidemic subjects, also after adjusting for age, ferritin and CRP. Similar results were found also in the work of Zhu and collaborators where they suggested that a higher level

of hepcidin was associated with a higher risk of dyslipidemia in children, which might contribute to the increased risk of atherosclerosis and related CVD in adulthood [80].

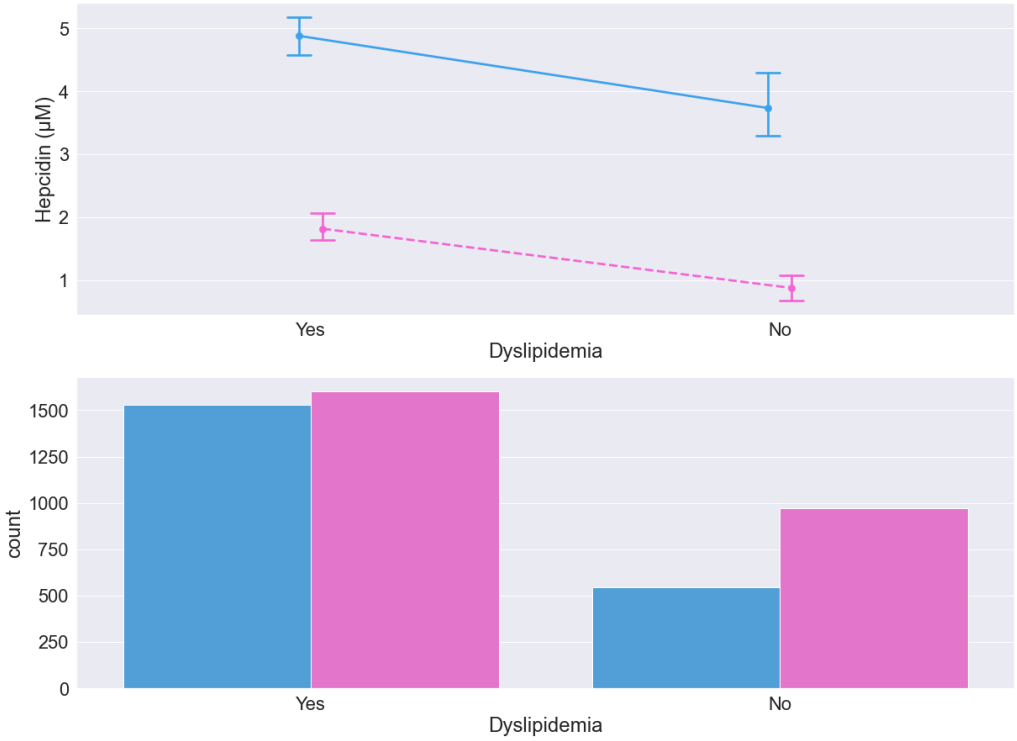


Figure 24: Variation of hepcidin concentration in subjects with and without dyslipidemia. Males are indicated by a continuous blue line, females by a dotted pink line. Males unadjusted and adjusted p<0.001; females unadjusted and adjusted p<0.001.

Serum hepcidin reference ranges

Reference ranges for serum hepcidin concentration, stratified by 10-year age groups and sex, were constructed using geometric mean with 95% of CI for each category. A reference subset was selected from the CHRIS population by excluding subjects with the following criteria:

- FT<30 ng/ml
- ALT>50 U/l
- CRP>1 mg/dl
- daily alcohol intake>80 g for men
- daily alcohol intake>40 g for women
- presence of liver disease
- BMI>30 kg/m^2

- HGB<13 g/dl for males
- HGB<12 g/dl for females
- presence of *HFE* C282Y in homozygosity
- compound heterozygotes for *HFE* C282Y/H63D
- presence of diabetes mellitus
- particular pharmacological therapy (e.g. thyroid disease drugs, diabetes medication, metabolic syndrome drugs, blood pressure therapy).

A total of 1216 subjects (831 males and 385 females) passing set criteria were included in the reference subset. Serum hepcidin concentrations lower than LOD (0.5 nM) were observed for both men (N=73) and women (N=94). The lowest geometric mean of hepcidin concentration (0.78 nM) was found in the category of females 18 to 29 years old, whereas the highest geometric mean of concentration (4.08 nM) was observed in males aged between 40 to 49 years old. Hepcidin reference ranges measured in the subset of CHRIS population were lower and less wide than previously published ranges [81]. However, data presented in this work, as well as data presented by Galesloot, showed that hepcidin concentrations are lower for premenopausal than for postmenopausal women but are almost constant over age in men. Differences in reference ranges could be related to difficulties on the standardization of the methods and differences between assays. Indeed, generally immunochemical-based methods overestimates hepcidin-25 levels because most antibodies are not able to detect solely the bioactive isoform [37]. Table 7 shows the reference range of serum hepcidin-25 concentration in men and women at 6 different age groups, obtained by the LC-MS/MS approach used in our project (see *Serum Hepcidin Assay* section).

	Male (N=831)				Female (N=385)			
			95% reference range				95% reference range	
Age class	N	hepc25 GM	lower CI	upper CI	N	hepc25 GM	lower CI	upper CI
18-29	239	2,817	2,375	3,342	112	0,7882	0,5748	1,081
30-39	149	2,855	2,274	3,585	57	0,9049	0,5695	1,438
40-49	184	4,086	3,373	4,949	58	1,039	0,6694	1,614
50-59	149	3,547	2,855	4,407	92	2,267	1,662	3,093
60-69	75	3,207	2,335	4,405	42	2,939	1,901	4,544
>=70	35	3,029	1,935	4,741	24	3,486	2,12	5,731

Table 7: Reference ranges for serum hepcidin-25 (nM) per 10-year age group for men and women in the reference subset. These data were obtained by a LC-MS/MS approach. (GM: geometric mean; CI: confidence interval).

Genetic determinants of hepcidin-25

Genome-wide association analysis of hepcidin-25 was performed in the CHRIS participants, in order to study the association of SNVs with serum hepcidin. Six variants were chosen for the association analysis in order to replicate what emerged in a previous hepcidin-25 GWAS [82]. Figure 25 shows the six selected variants colored along with all variants within 1Mbp. As demonstrated by Galesloot and colleagues [82], these analyses do not reveal genetic determinants of serum hepcidin-25, in particular, the association between hepcidin-25 and these six selected variants were not significant.

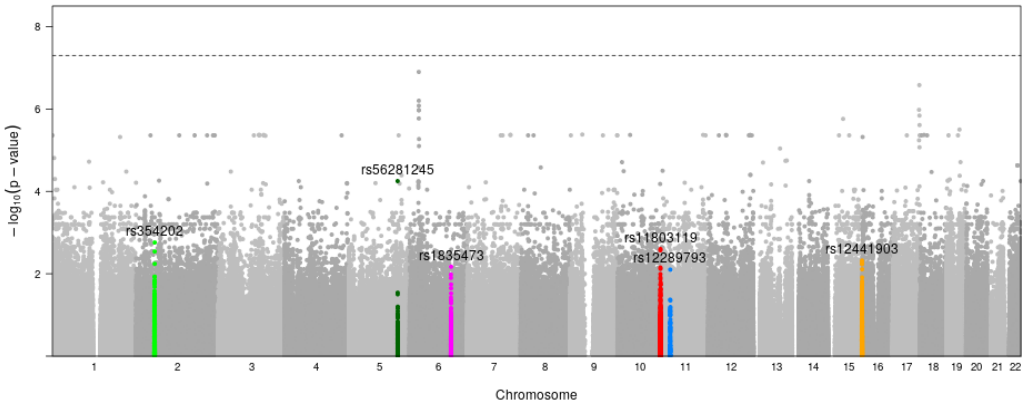


Figure 25: Manhattan Plot of the association analysis between hepcidin-25 and six selected variants (Horizontal dotted line indicates the significant threshold).

Single variant association analysis was performed also to investigate the association between hepcidin/ferritin ratio and selected SNPs. Data emerged from these analyses are consistent with previously identified associations [55, 82]. Indeed, the

association of rs1800562 with hepcidin/ferritin ratio was considered as significant (Figure 26). The rs1800562 in *HFE* gene is a well-known iron-related SNV and explains ~1% of serum iron variation. In addition, Galesloot's work showed that rs1800562 had a stronger signal with the ratio hepcidin/ferritin compared to the ratio hepcidin/TSAT, suggesting that this SNV have a larger influence on hepcidin response to body iron stores than on hepcidin response to circulating iron [82].

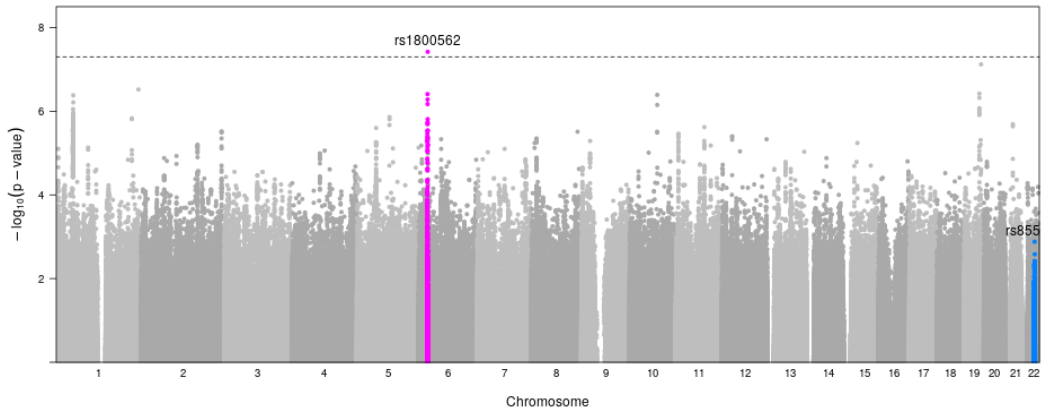


Figure 26: Manhattan Plot of the association analysis of SNPs with hepcidin/ferritin ratio (Horizontal dotted line indicates the significant threshold).

HEPCIDIN-24 ANALYSIS IN THE CHRIS POPULATION

Serum hepcidin-24 levels were also measured in the cohort of CHRIS population. Hepcidin-24 was detectable in 725 of 4644 analyzed subjects (Figure 27), unlike hepcidin-25, which was detectable in 3429 individuals (Figure 14).

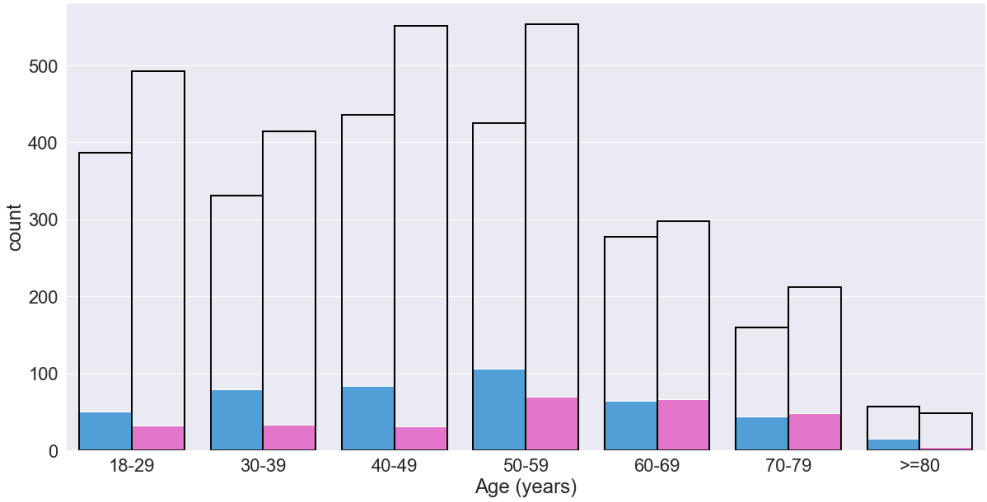


Figure 27: Distribution of detectable hepcidin-24 by age and sex (blue for males and pink for females).

Figure 28 shows the histograms of hepcidin-24 for a better visualization of its distribution in the whole population (on the top) and only in detectable values (on the bottom).

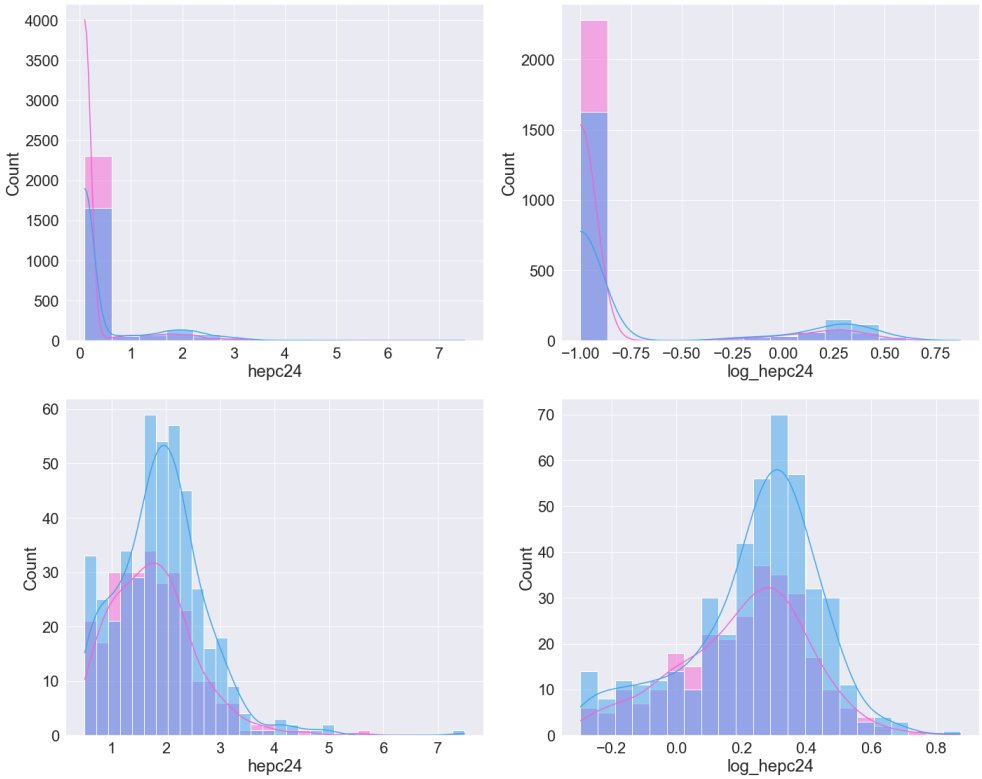


Figure 28: the histograms represent the distribution of hepcidin-24 values in the whole population with its respective log-transformed values (on the top). Only hepcidin-24 detectable values followed by its log-transformed concentrations (on the bottom). Pink color indicates females while blue indicates males.

Subjects with detectable hepcidin-24 are older and have an higher body iron status (as reflected by transferrin saturation and ferritin levels) and hepcidin-25 levels if compared to subjects with undetectable hepcidin-24 (Table 8) (Figure 29).

Trait	Units	Hepcidin-24 undetectable (N=3919)			Hepcidin-24 detectable (N=725)			p°
		N	Median	CI	N	Median	CI	
Male sex	%	1631	41.6	-	442	60.9	-	**
Age	Years	3919	45.3	44.79-45.8	725	52.15	50.95-53.34	****
BMI	Kg/m2	3849	24.7	24.55-24.84	707	26	25.65-26.34	****
HGB	g/dl	3915	14.6	14.55-14.64	725	15.1	15.01-15.18	****
HbA1c	%	3916	5.6	5.58-5.61	725	5.7	5.66-5.73	****
MCV	Fl	3915	91.3	91.15-91.44	725	91.4	91.07-91.72	NS
MCH	pg	3915	30.7	30.64-30.75	725	30.8	30.67-30.92	*
MCHC	g/dl	3915	33.6	33.56-33.63	725	33.7	33.63-33.76	**
Iron	µg/dl	3917	109	107.74-110.25	724	112	109.23-114.76	**
TIBC	µg/dl	3919	347	345.13-348.86	725	330	326.63-333.36	****
Ferritin	ng/ml	3919	71.5	67.49-75.5	725	168.5	156.65-180.34	****
Transferrin	mg/dl	3919	278	276.5-279.49	725	264	261.31-266.68	****
TSAT	%	3917	27.9	27.55-28.24	724	29.6	28.83-30.36	****
ALT	U/l	3919	17	16.57-17.42	725	21	19.94-22.05	****
Hepcidin-25	nM	3919	2.03	1.89-2.16	725	7.79	7.34-8.23	****
Hepcidin-24	nM	3919	<0.5	-	725	1.81	1.75-1.87	****
Daily alcohol intake (DAI)	g	1905	11.14	10.46-11.81	405	13.71	12.18-15.23	NS
Cholesterol	mg/dl	3919	208	206.72-209.27	725	213	209.92-216.07	**
HDL cholesterol	mg/dl	3919	64	63.46-64.53	724	62	60.79-63.2	****
Triglycerides	mg/dl	3919	85	83.08-86.91	725	93	87.25-98.74	****
Glucose	mg/dl	3919	90	89.57-90.42	725	94	92.99-95	****
CRP	mg/dl	3909	0.12	0.10-0.13	725	0.14	0.09-0.18	****
Creatinine	mg/dl	3919	0.87	0.86-0.87	725	0.93	0.91-0.94	****

Table 8: Characteristics of subjects stratified according to hepcidin-24 detectable.
**p<0.05, **p<0.01, ****p<0.0005.*
BMI: Body Mass Index; CRP: C-Reactive Protein; HGB: hemoglobin; HbA1c: glycated hemoglobin; MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; TIBC: total iron biding capacity; TSAT: transferrin saturation; ALT: alanine aminotransferase; MCHC: mean corpuscular hemoglobin concentration; N: Sample size; CI: 95% confidence interval; NS: not significant.

As mentioned before, hepcidin-24 seems to be elevated during inflammation [6]. There is a significant difference between the two groups in CRP, with an increase of 16.6% from undetectable to detectable hepcidin-24 (from 0.12 mg/dl to 0.14 mg/dl). As shown in Figure 29 the difference is most evident in females, suggesting a putative role of hepcidin-24 in inflammatory conditions.

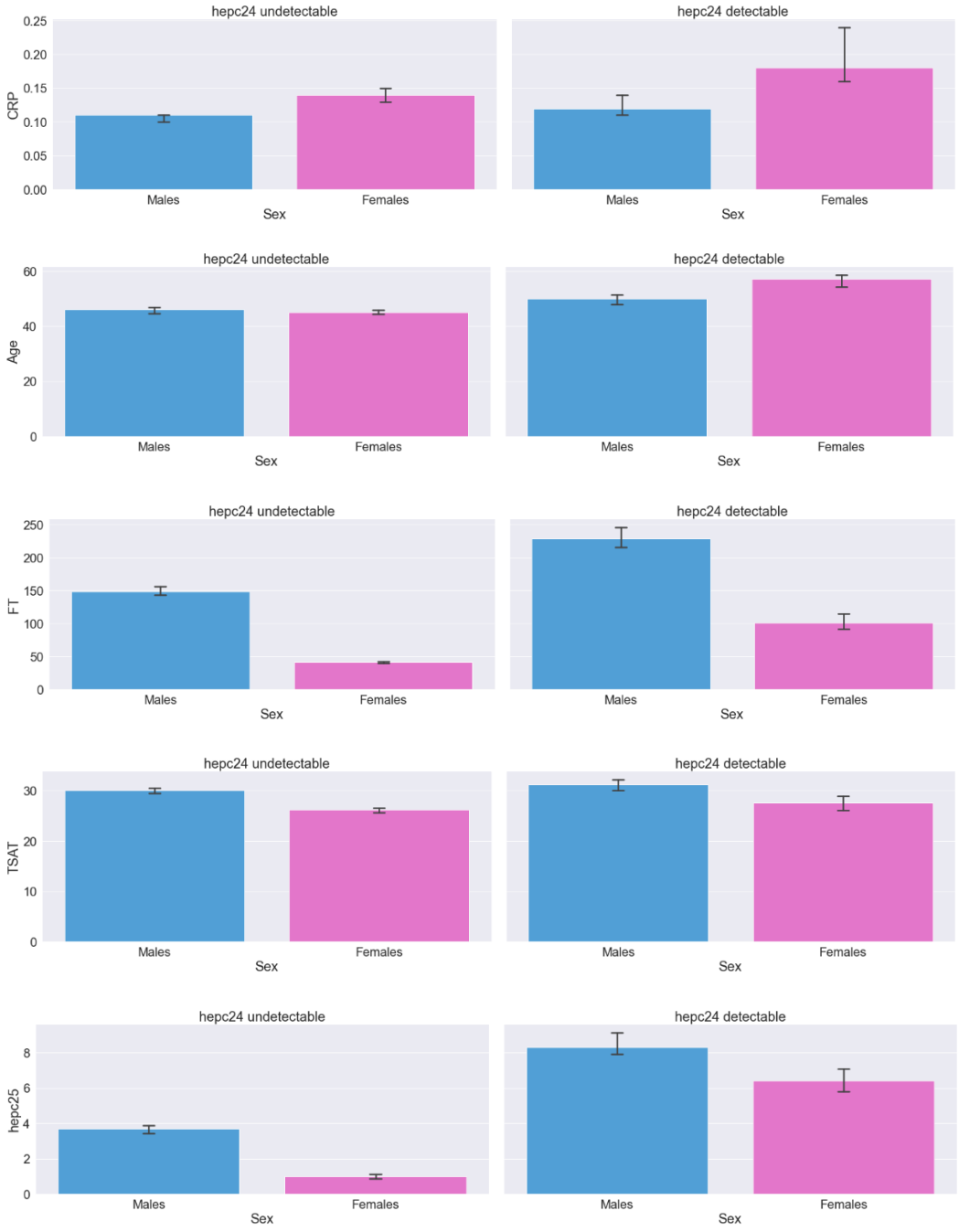


Figure 29: Differences of CRP, age, FT, hepcidin-25 and TSAT in hepcidin-24 undetectable and detectable groups by sex.

Spearman correlation test showed that the two hepcidin isoforms were significantly and positively correlated in males (r_s=0.418, p<0.001) and females (r_s=0.389, p<0.001). Table 9 summarizes Spearman correlation results.

Traits	Males	Females
Ferritin	0.265*	0.291*
TSAT	0.053*	0.042*
Age	0.097*	0.177*
Iron	0.045*	-0.01
Transferrin	-0.05*	-0.15*
TIBC	-0.05*	-0.15
ALT	0.067*	0.098*
CRP	0.074*	0.092*
HbA1c	0.067*	0.129*
MCV	-0.01	0.053*
Daily alcohol intake	0.038	0.041
BMI	0.066*	0.116*
Cholesterol	0.05*	0.054*
Triglyceride	0.031	0.073*
HDL	0.022	-0.01
Creatinine	0.021	0.009
HGB	-0.01	0.056*
MCH	-0.0	0.046*
MCHC	0.01	0.008
Glucose	0.078*	0.128*
Hepcidin-25	0.418*	0.389*

*Table 9: p-value of Spearman correlation of hepcidin-24 for males and females. *Spearman's r with p-value < 0.05.*

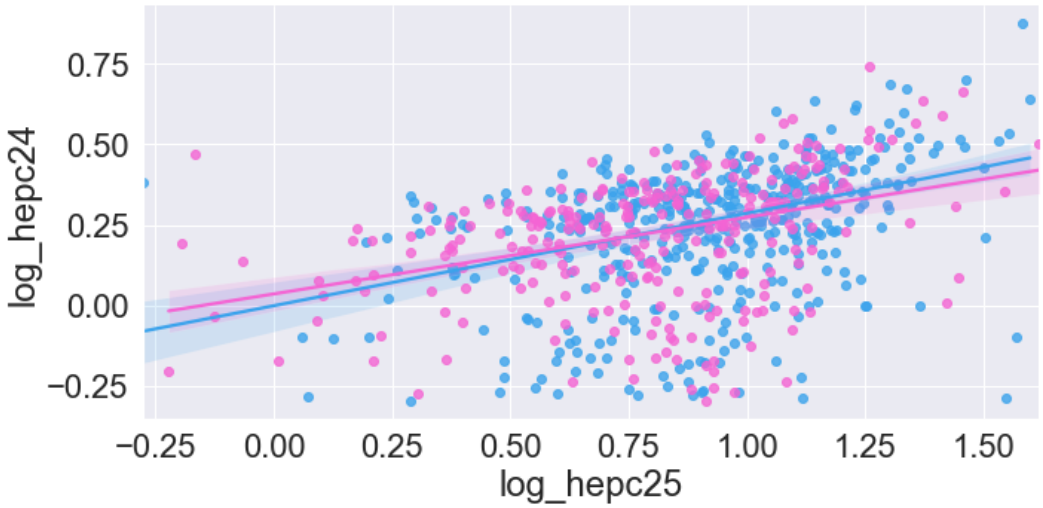


Figure 30: Correlation plot between hepcidin-24 and hepcidin-25 (logarithmic scale). Pink color indicates females while blue indicates males.

At simple linear regression analysis hepcidin-24 correlated significantly with ferritin, BMI and hepcidin-25 in men, and ferritin and hepcidin-25 in women (Table 10). Figure 30 shows the linear regression between hepcidin-24 and hepcidin-25 for both males and females.

Trait	Males		Females	
	β	p	β	p
Log Ferritin	0.1057	**	0.0708	****
BMI	0.0863	**	-	-
Log hepcidin-25	0.1168	****	0.094	****

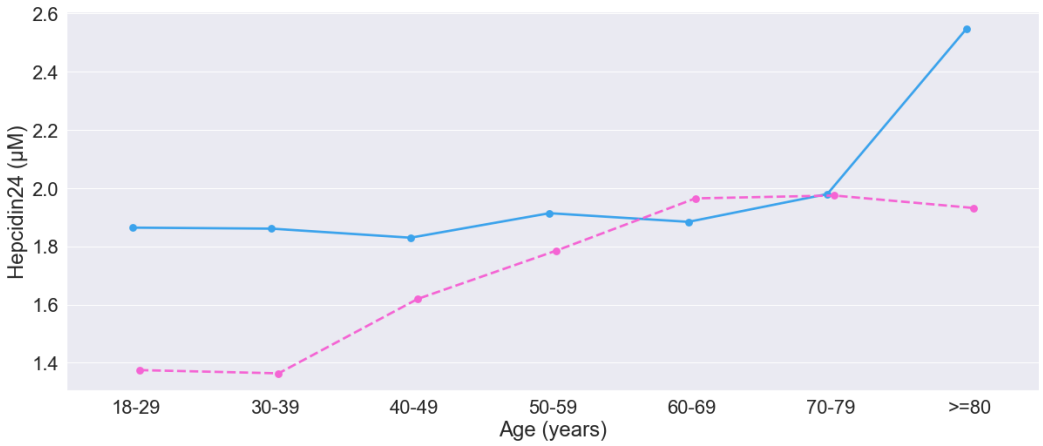
*Table 10: Single linear regression analysis of serum hepcidin-24 by sex. **p<0.01, ****p<0.0005.*

At multiple linear regression analysis hepcidin-25 and age resulted as independent significant predictors of hepcidin-24 in males (with an adjusted R-squared = 0.127). In females, hepcidin-25, age and ferritin were the independent predictors of hepcidin-24 (with an adjusted R-squared = 0.137) (Table 11).

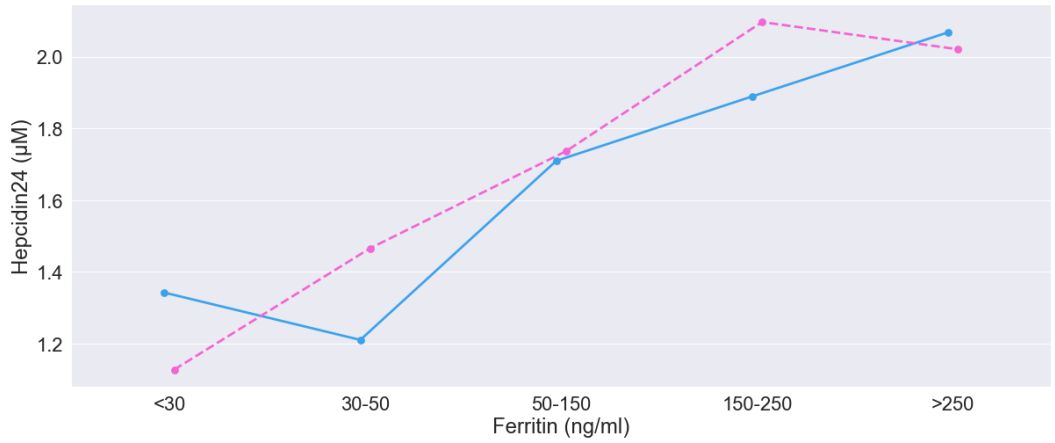
Trait	Males		Females	
	β	p	β	p
Log Ferritin	0.0249	NS	0.024	*
BMI	-0.0045	NS	-	-
Log hepcidin-25	0.1636	****	0.1183	****
Age	0.0375	**	0.0157	*

*Table 11: Multiple linear regression analysis of serum hepcidin-24 by sex. *p<0.05, ****p<0.0005, NS: not significant.*

Figure 31 reported the variation of hepcidin-24 in the CHRIS population according to different ranges of age and iron status (reflected by ferritin levels), respectively.



Hepc-24 (nM) Males	1.864 (1.66-1.96)	1.86 (1.72-2.19)	1.82 (1.72-2.03)	1.91 (1.71-2.05)	1.88 (1.77-2.03)	1.97 (1.49-2.21)	2.54 (1.26-2.74)	*p<0.001
Hepc-24 (nM) Females	1.37 (1.1-1.6)	1.36 (1.23-1.5)	1.61 (1.03-1.92)	1.78 (1.57-1.97)	1.96 (1.72-2.2)	1.97 (1.58-2.16)	1.93 (1.67-3.12)	*p<0.001



Hepc-24 (nM) Males	1.34 (1.05-1.63)	1.21 (0.9-2.08)	1.7 (1.46-1.83)	1.88 (1.77-2.03)	2.06 (1.88-2.2)	*p<0.001
Hepc-24 (nM) Females	1.12 (0.96-1.56)	1.46 (1.25-1.69)	1.73 (1.57-1.87)	2.09 (1.76-2.29)	2.02 (1.1-2.67)	*p<0.001

*Figure 31: Behavior of hepcidin-24 in the CHRIS population according to different ranges of age (top panel) and ferritin (bottom panel). Males are indicated by continuous blue line, females by a pink dotted line. Values are reported as median with 95% CI. * p-value obtained by ANOVA with polynomial contrasts for linear trend.*

Hepcidin-24 and hepcidin-25 levels were also reported in parallel, after stratification for ferritin levels (Figure 32).

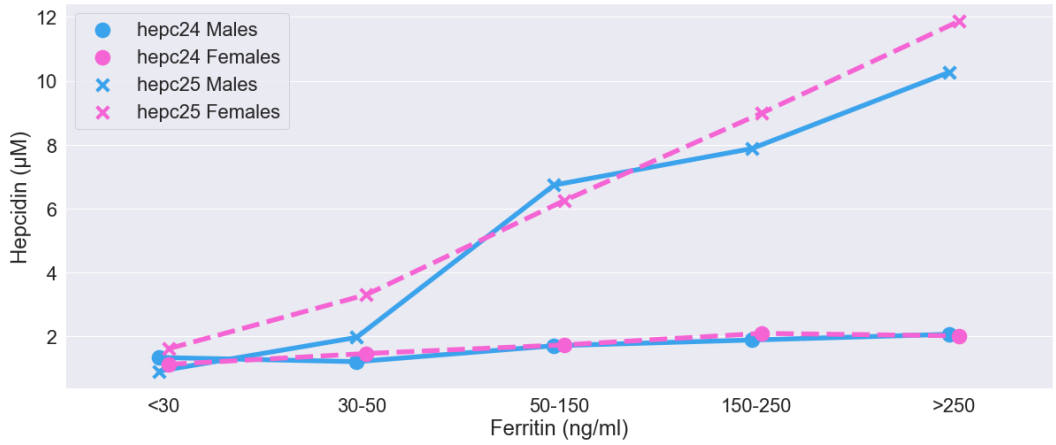


Figure 32: Behavior of hepcidin-24 and hepcidin-25 in males and females according to ferritin levels. Significant differences in hepcidin-25 were verified in both genders. Values are reported as median with 95%. p<0.001 obtained by ANOVA with polynomial contrasts for linear trend.

In Figure 33 the relative percentages of hepcidin-25 and hepcidin-24 were reported, according to increasing ferritin levels in males and females, respectively. The relative percentage of hepcidin-24 progressively and significantly decreased with increasing ferritin levels, in men and women. These observations suggest that in subjects with iron deficiency condition the few hepcidin-25 produced may be efficiently degraded to keep the iron bioactive peptide as low as possible to maximize intestinal iron absorption. In contrast, in subjects with adequate or high iron status hepcidin-25 degradation may proceed less efficiently to keep a normal iron balance or to prevent dangerous iron overload. Moreover, these results suggest that the relative contribution of hepcidin-24 to total serum hepcidin is not negligible at population level. In the total population, mean hepcidin-24 levels were about 27% of the total hepcidin concentration (here defined as the sum of hepcidin-24 and hepcidin-25 concentrations).

All the analysis carried out (Spearman correlation and linear regression) showed a positive relationship between hepcidin-24 and hepcidin-25, both in males and females, suggesting that hepcidin-24 could represent a constitutive degradation product of hepcidin-25. To the best of our knowledge, the biological meaning of smaller isoforms is still unclear, therefore it is unknown whether or not the

processing of hepcidin-25 cleavage is an actively regulated mechanism. Therefore, hepcidin-24 could represent either a final degradation product of hepcidin-25 and/or a functional peptide whose production is actively regulated.

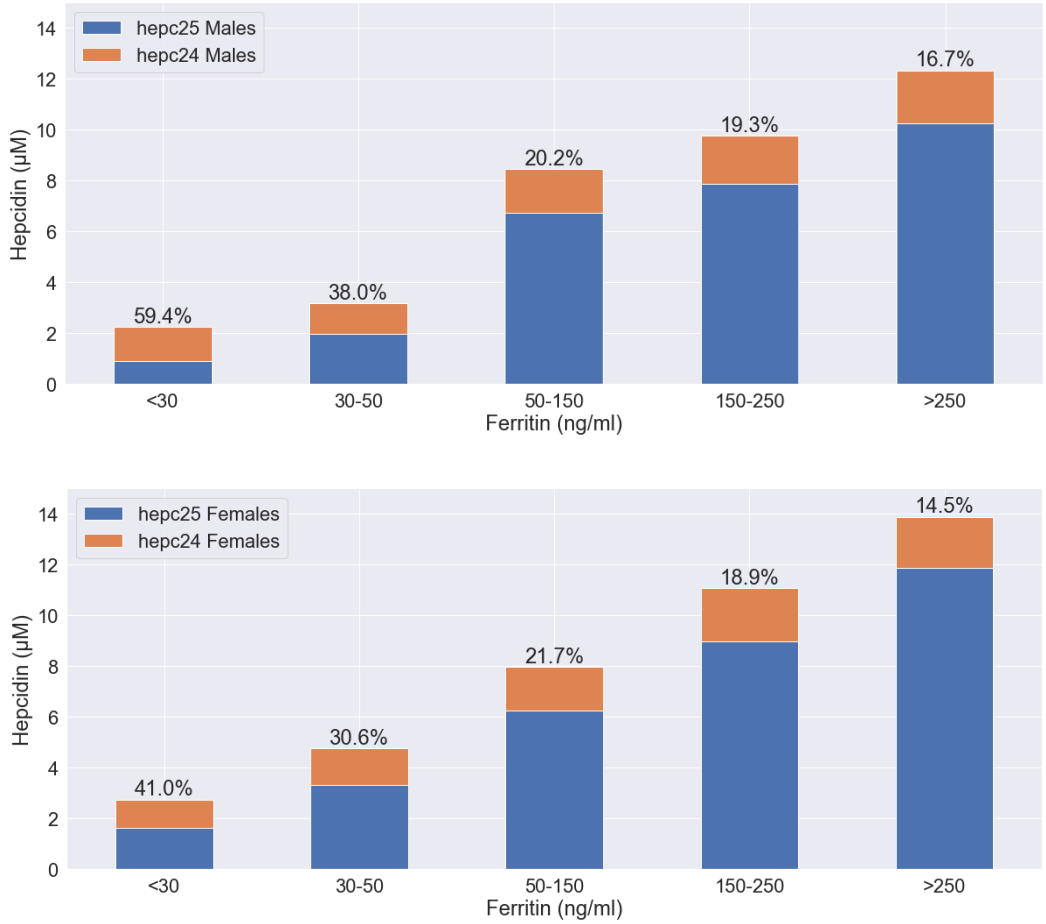


Figure 33: Relative percentages of hepcidin-25 and hepcidin-24 according to increasing ferritin levels in males (upper panel) and females (lower panel). The percentage value represents the proportion of hepcidin-24 on total hepcidin (calculated as hepcidin-25 + hepcidin-24). $p<0.001$ in both genders obtained by ANOVA with polynomial contrasts for linear trend.

Statistical analyses regarding hepcidin-20 were not performed because only a negligible number of individuals had detectable levels of this isoform (Figure 34).

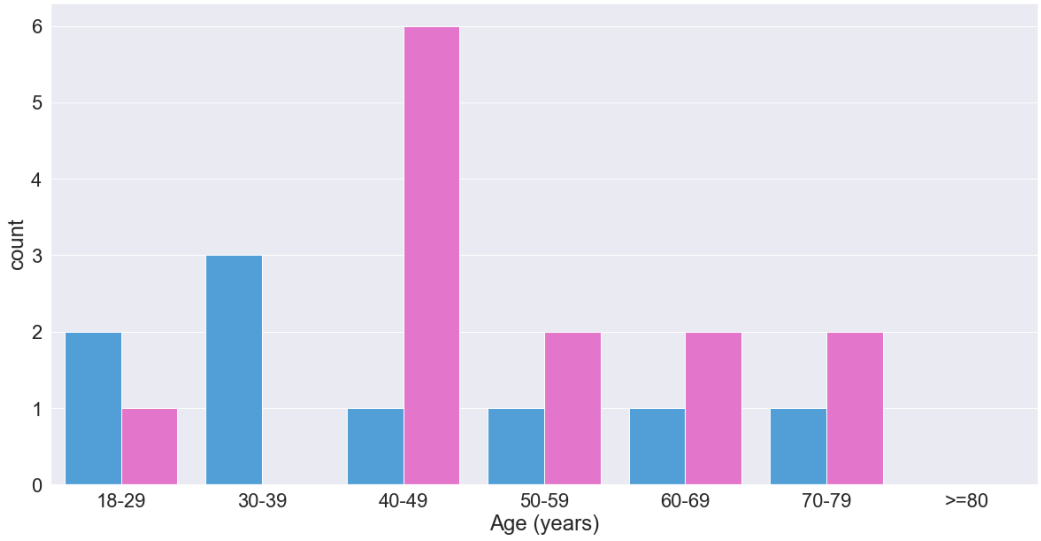


Figure 34:Distribution of hepcidin-20 detectable (>0.5 nM) by age and sex. Pink color indicates females while blue indicates males.

sTfR AND ERFE ANALYSIS IN THE CHRIS POPULATION

Serum sTfR was measured by a commercially available ELISA method in 4635 subjects of the CHRIS population. The statistical analysis of ERFE and sTfR data is still ongoing, therefore it was possible to report in this manuscript only preliminary results. In particular, some ERFE preliminary analysis was kindly provided by the EURAC Center of Research (Bolzano, Italy).

Figure 35 shows histograms with the distributions of serum sTfR and ERFE values in the CHRIS population.

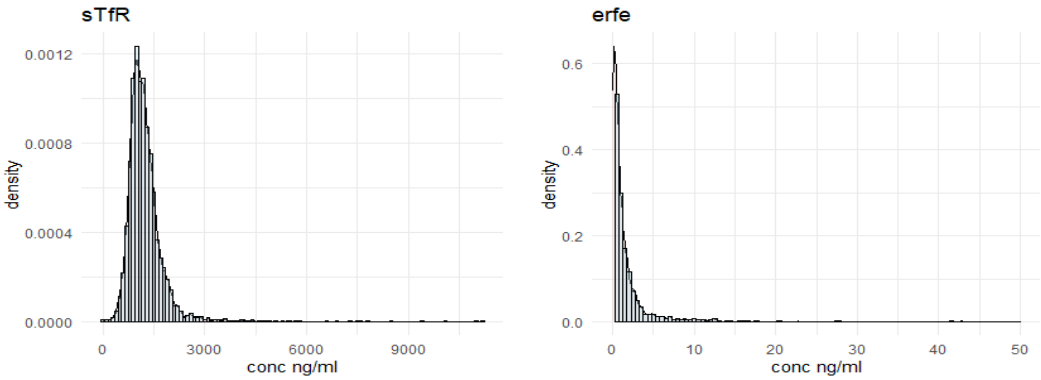


Figure 35: sTfR and ERFE distribution in the whole population.

Serum ERFE was measured by an ELISA approach in 3727 serum samples of the CHRIS population (1680 males and 2047 females; age range 18-93). A total of 781 individuals (336 males and 445 females) had undetectable ERFE concentrations (<0.16), which represents the 21% of the entire population. The overall median serum ERFE concentration was 0.67 ng/ml (IQR: 0.22-1.69).

Figure 36 and Figure 37 show the trend of the hepcidin and sTfR classes in relation to ERFE quintiles.

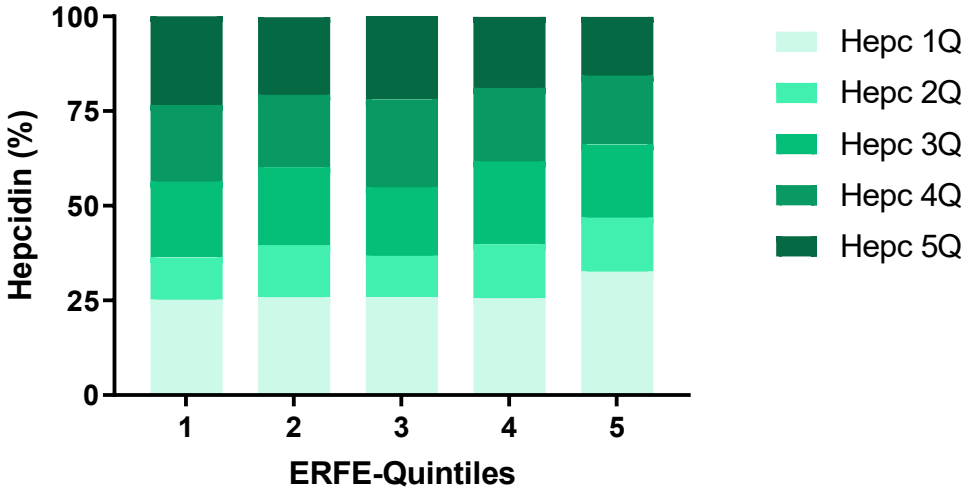


Figure 36: Representation of the trend of hepcidin classes in relation to ERFE quintiles. (1Q-5Q: first quintile to fifth quintile)

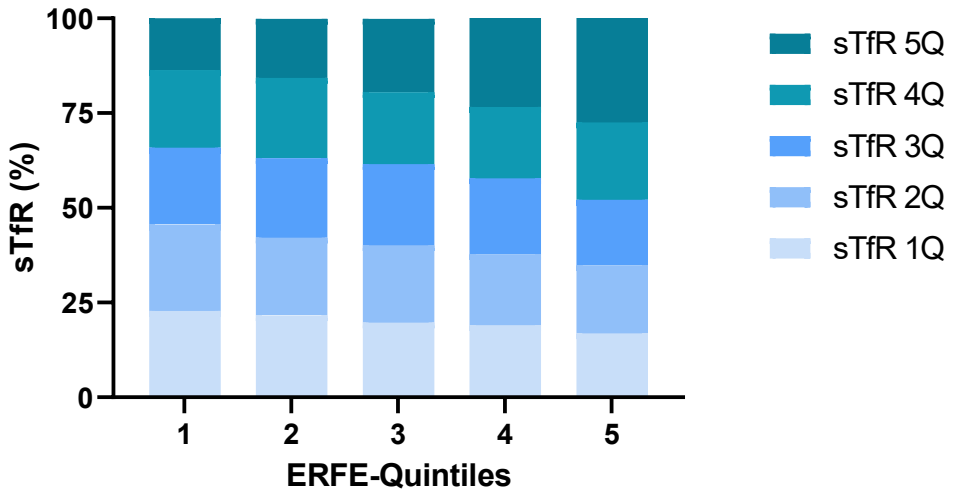


Figure 37: Representation of the trend of sTfR classes in relation to ERFE quintiles. (1Q-5Q: first quintile to fifth quintile)

Serum ERFE distribution by age and sex are reported in Table 12.

Age Class	N	Males	N	Females
18-29	333	0.63 [0.17-1.65]	430	0.5 [0.16-1.57]
30-39	266	0.62 [0.26-1.4]	323	0.64 [0.17-1.85]
40-49	373	0.6 [0.22-1.38]	464	0.76 [0.22-1.97]
50-59	334	0.79 [0.31-1.61]	411	0.63 [0.21-1.68]
60-69	209	0.73 [0.25-1.7]	233	0.72 [0.33-1.84]
70-79	127	0.83 [0.3-1.8]	155	0.92 [0.41-1.63]
>=80	38	0.88 [0.46-1.95]	31	0.68 [0.16-1.6]

Table 12: Serum ERFE levels by age and sex. Values are represented as median and IQR.

As we can see in Figure 38, age behaves differently in both sexes with a much more significant effect in females ($p < 0.001$) than in males ($p < 0.05$). Nevertheless, the trend of ERFE seemed to have a less significant age- and gender-dependent variation, if compared with hepcidin one, and only shows a slight tendency to increase with age, a fact more evident in males than in females (Table 12).

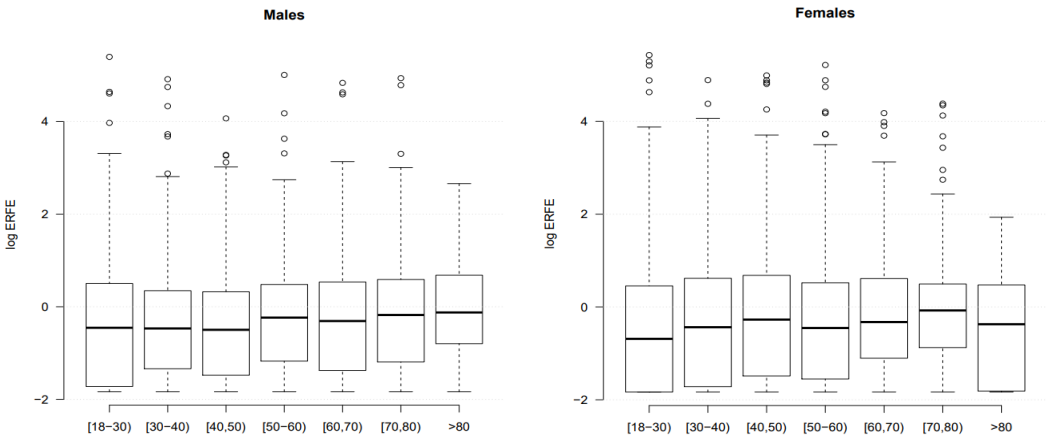


Figure 38: Age and sex variations of serum ERFE in the whole population. ERFE values are reported as median in logarithmic scale. Females to the right, males to the left.

Spearman correlation analysis was performed for hepcidin (20, 24 and 25 isoforms), ERFE and sTfR variables, in the entire population. ERFE showed a negative correlation with hepcidin-25 (r_s = -0.073; $p < 0.001$) and a positive correlation with sTfR (r_s = 0.11; $p < 0.001$) (Figure 39).

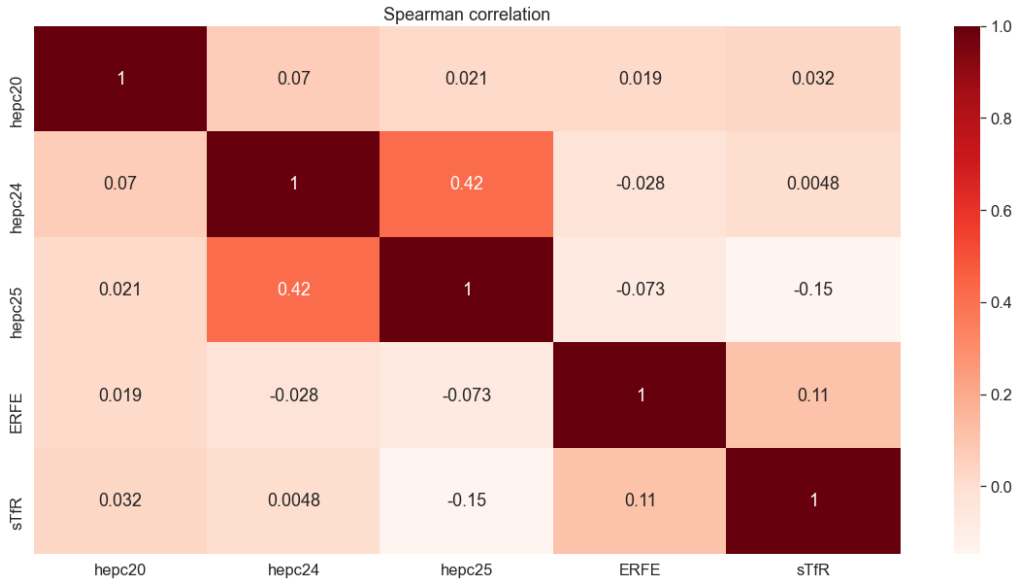


Figure 39: Spearman correlation with hepcidin (20, 24, 25 isoforms), ERFE and sTfR. Correlation strength of $|r_s|$ coefficient: 1-0.8 very strong; 0.79-0.6 strong; 0.59-04 moderate; 0.39-0.2 weak; 0.19-0 very weak.

To study the relationship between serum ERFE and other parameters, simple linear regression analyses were performed, using age and sex as covariate (Table 13). HGB (β = -0.1692) and RBC (β = -0.1858) resulted to have the strongest association

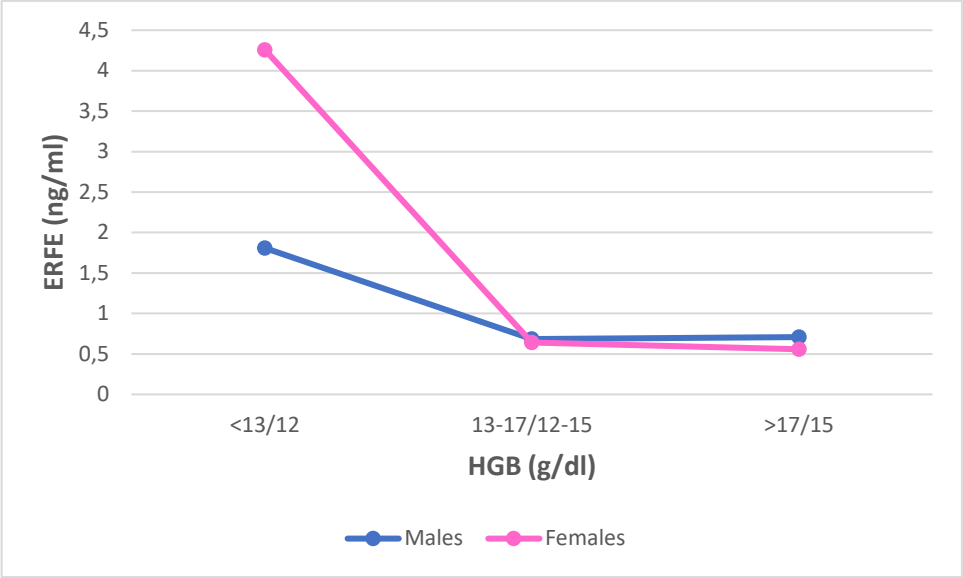
with ERFE (with negative β coefficients). These results are consistent with a previous work, where serum ERFE concentrations correlates negatively with HGB in pediatric patients with iron deficiency anemia [83].

Trait	β	p
Age	0.0069	***
HGB	-0.1692	***
MCV	-0.0307	***
MCH	-0.0738	***
MCHC	-0.056	*
RBC	-0.1858	*
CRP	-0.017	NS
sTfR	0.0004	***
Ferritin	-0.0005	*
Iron	-0.0035	***
Transferrin	0.002	***
TIBC	0.0016	***
ALT	0.0031	NS
Hepcidin-25	-0.0209	***
Hepcidin-25 detectable	-0.2762	***
Creatinine	-0.0624	NS
HbA1c	0.0534	NS
Cholesterol	-0.0013	*
HDL	-0.0032	NS
Triglyceride	-0.0001	NS
BMI	-0.0004	NS

*Table 13: Single linear regression analysis of serum ERFE. *p<0.05, ***p<0.001, NS: not significant.*

In order to better study the negative relation between hemoglobin and ERFE, the population was stratified in three classes according to hemoglobin values corresponding to low hemoglobin count (HGB<13 g/dl in males and <12 g/dl in females), normal hemoglobin count (13≤HGB≤17 g/dl in males and 12≤HGB≤15 g/dl in females) and high hemoglobin count (HGB>17 g/dl in males and >15 g/dl in females) as shown in

Figure 40. It is evident how the ERFE concentrations decrease sharply in normal hemoglobin count, in both genders.



ERFE (ng/ml) Males	1.80 (0.63-6.71)	0.68 (0.24-1.61)	0.7 (0.31-1.4)
ERFE (ng/ml) Females	4.25 (1.32-8.04)	0.64 (0.18-1.69)	0.55 (0.25-1.39)

Figure 40: Serum ERFE in groups of individuals classified according to different hemoglobin levels. Values are reported as median and IQR. $p<0.05$ in males and $p<0.001$ in females.

Reference ranges for serum ERFE concentration, stratified by 10-year age groups and sex, were constructed using median and interquartile range for each category. A reference subset was selected from the CHRIS population by excluding subjects with comorbidities.

	Males			Females		
		IQR			IQR	
Age class	**ERFE median**	**1st Q**	**3rd Q**	**ERFE median**	**1st Q**	**3rd Q**
18-29	0.62	0.17	1.6	0.48	0.16	1.25
30-39	0.64	0.26	1.48	0.49	0.16	1.31
40-49	0.56	0.22	1.27	0.51	0.16	1.6
50-59	0.76	0.24	1.56	0.54	0.18	1.25
60-69	0.73	0.21	1.83	0.82	0.39	2.37
70-79	0.63	0.16	1.36	0.88	0.46	1.5
>=80	0.97	0.59	1.72	1.11	0.29	1.66

Table 14: proposed ERFE reference ranges (ng/ml) per 10-year age group for men and women in the reference subset. (IQR: interquartile range; 1st Q: first quartile; 3rd Q: third quartile).

A total of 1719 subjects were included in the reference subset. The lowest median of ERFE concentration (0.48 ng/ml) was found in the category of females 18 to 29 years old, whereas the highest median of concentration (1.11 ng/ml) was observed

in the group of females with more than 80 years old (Table 14). The overall serum ERFE reference range was 0.21-1.53 ng/ml.

Finally, the population was stratified in three classes according to ERFE levels: ERFE concentrations bellow the reference range (<0.21 ng/ml), ERFE concentrations in the refence range (0.21-1.53 ng/ml), and above the refence range (>1.53 ng/ml). Figure 41 shows that the sTfR concentrations increased progressively with ERFE increasing and differ significantly among the three groups ($p<0.001$). Figure 42, on the contrary shows a significant reduction in hepcidin levels associated to ERFE increasing ($p<0.001$).

All the preliminary results here presented reveal a strong association of serum ERFE with hemoglobin and RBCs. However, on the contrary to what expected, the correlation with hepcidin appears to be very weak. The analysis of hepcidin variation according to different levels of ERFE and the ERFE variation according to different hemoglobin levels could suggest a non-linear relationship between these two hormones. There could be a threshold level of ERFE where the transcription of hepcidin is inhibited, as a sort of on-off mechanism. Another limit of this study is related with the performance of serum ERFE assay. Only the 77% of samples passed the quality control as the remaining 23% was not taken in consideration, because presented a CV > 14% in repeated measurements also after re-assay. This ERFE kit has been extensively studied in a previous work, where the authors observed the same problem. They showed that the coefficients of variations were 17% and 23% for inter-assay variation of replicate measurement and 32% and 26% for the intra-assay variation in the low (5 ng/ml) and high ERFE (35 ng/ml) controls, respectively [84]. Therefore, reproducibility of the assay needs to be improved. The performance of the assay could be ameliorated with some precautions, such as minimize freezing and thawing cycles, keep the ERFE standard at -80 ˚C instead of 4 ˚C, ensure better conservation of the kits even during shipment.

These results, thus promising have to be taken with caution and need to be confirmed and extended.

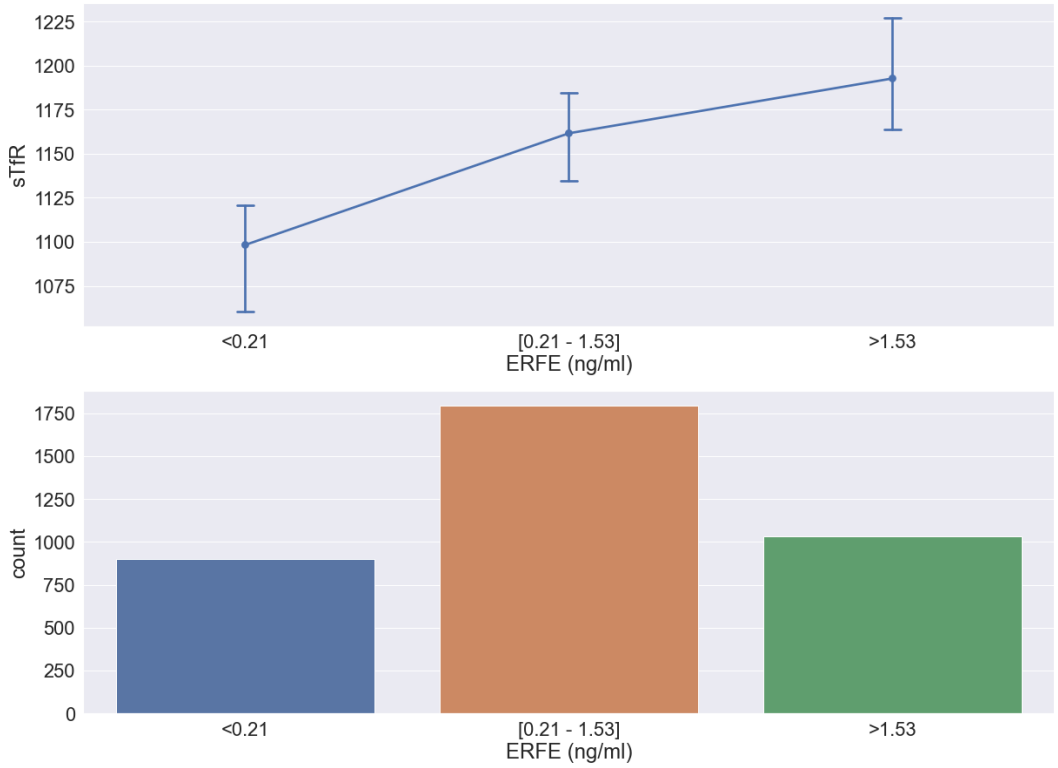


Figure 41: Serum sTfR in groups of individuals classified according to different ERFE levels. Values are reported as median and CI.

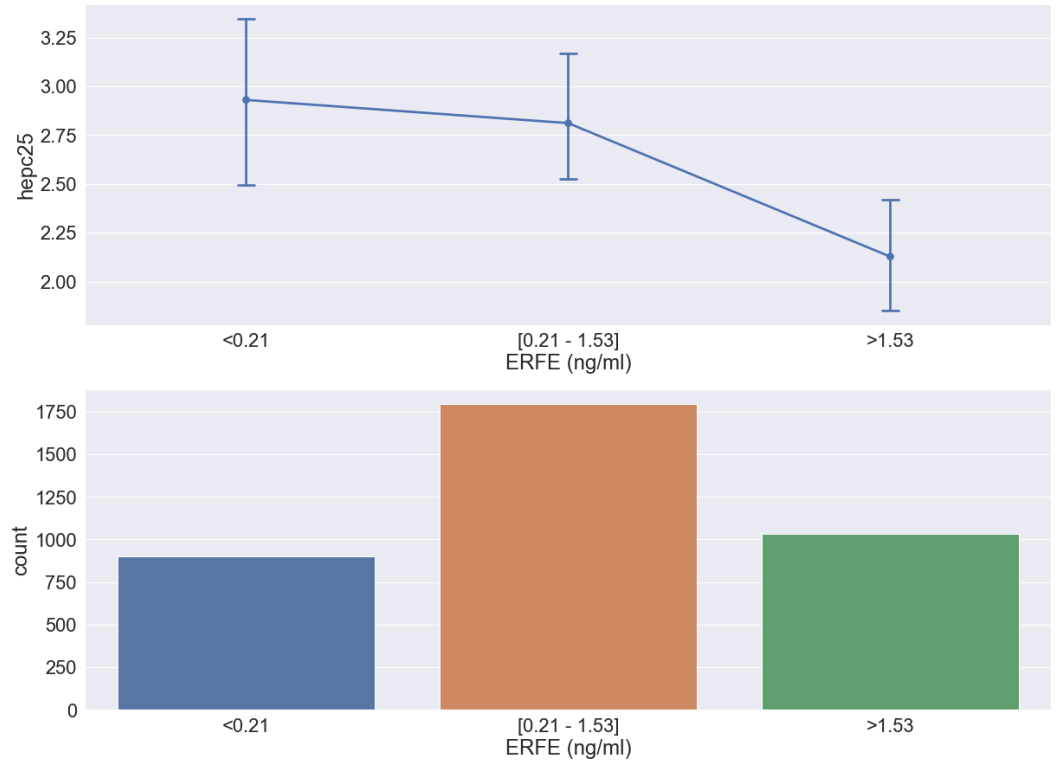


Figure 42: Serum hepcidin in groups of individuals classified according to different ERFE levels. Values are reported as median and CI.

CONCLUSIONS

Serum hepcidin-25, erythroferrone and soluble transferrin receptor were measured in the large cohort of CHRIS population, through an LC-MS/MS approach (for hepcidin) and ELISA assays (for ERFE and sTfR).

Our study confirmed previous observations regarding the trend of hepcidin in the general population [55, 85]. A substantial stability of hepcidin in males and greater variability in females was confirmed at the population level. Moreover, iron deposits (whose ferritin is a surrogate in the absence of intercurrent inflammation) proved to be the main determinants of hepcidin. An association also emerged between hepcidin and the circulating iron pool (serum iron and TSAT), not present in previous studies [55]. A decrease in hepcidin levels was confirmed in the elderly (> 80 years), in agreement with the reduction of the individual's iron assets (decrease in ferritin and TSAT). Although an increase in hepcidin levels has been considered a possible determinant of anemia in the elderly (as a possible effect of inflammaging) [86], this does not seem to occur at the general population level. Specific analyses in anemic subjects are planned to clarify this point. Moreover, Hepcidin-25 concentrations increased significantly in women with metabolic syndrome, confirming an association between hepcidin and metabolic syndrome in females, as already shown in Martinelli et al. in 2012 [73].

We also analyzed the hepcidin trend in a subset of subjects without apparent confounding factors (FT<30 ng/ml; ALT>50 U/l; CRP>1 mg/dl; daily alcohol intake>80 g for men and >40 g for women; presence of liver disease; BMI>30 kg/m^2; HGB<13 g/dl for males and <12 g/dl for females; presence of *HFE* C282Y in homozygosity; compound heterozygotes for *HFE* C282Y/H63D; presence of diabetes mellitus; particular pharmacological therapy), in order to identify a “dynamic” reference range for hepcidin. This range has been confirmed to vary according to the age and gender of the subject, which could be useful in clinical practice. To date, the routine use of hepcidin as a diagnostic marker in clinical practice is still very limited, due to the lack of standardized techniques for its measurement and the difficulty of interpreting the results, which requires a center with adequate expertises.

By using the LC-MS/MS approach it was also possible to measure smaller isoforms of hepcidin-25. This study showed that the contribution of hepcidin-24 to the total serum hepcidin is not negligible at population level. Moreover, the relative percentage of hepcidin-24 progressively and significantly decreased with increasing ferritin levels, in men and women, suggesting that the degradation of hepcidin-25 could also be a regulated process according to iron need.

To the best of our knowledge, this study is the first that analyzed ERFE behavior in the general population. In addition to this, the population we used is very well characterized in terms of biochemical, hematological and anthropometric parameters, as very important erythropoietic (including sTfR) and iron (including hepcidin) markers are included.

Our proposed serum ERFE reference range (0.21-1.53 ng/ml) is lower than that found by Ganz (3-21 ng/ml) in the serum samples of 58 blood donors [31], and quite similar to that found by Appleby (0.12-1.25 ng/ml) in the plasma of 155 healthy volunteers, using the same commercially available ELISA kit [84]. Moreover, ERFE trend seemed to have a less significant age- and gender-dependent variation, if compared with hepcidin one, and only shows a slight tendency to increase with age, a fact more evident in males than in females.

Although our study is population based, and therefore any kind of cause-and-effect inference could not being done, the increase in ERFE levels in anemics subjects (median ERFE values of 1.8 in males and 4.25 in females) compared to non-anemics (median ERFE values of 0.68 in males and 0.64 in females) seems to confirm its role as a marker of erythropoietic activity in the general population as well as in pathological conditions, where it has already been well defined (e.g. beta-thalassemia) [29].

Preliminary ERFE correlation analyses indicated an apparently negligible negative association with hepcidin. We hypothesized that the relation between ERFE and hepcidin could not be linear, and as they are two hormones, there could be a threshold level of ERFE where the transcription of hepcidin is inhibited, as a sort of on-off mechanism. The lack of a clear correlation could also depend on some

technical issues, such as the large amount of undetectable values for hepcidin (26%) and ERFE (21%). Indeed, we have to consider that the experimental assay used for ERFE presented some drawbacks and its performance in terms of inter and intra assay CV are improvable, as also highlighted by Appleby and colleagues.

Data presented in this manuscript are very promising, particularly data concerning serum ERFE levels as, to the best of our knowledge, they have never been described before in such a large population. All the individuals involved in this part of the project were genotyped, and the genetic data of the CHRIS participants will be included in a Genome-Wide Association Study. Thus, this study will potentially provide interesting insights into better understanding the genetic determinants of ERFE and how these can influence the complex scenario connecting erythropoiesis and iron metabolism.

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