Peer Review File
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Reviewer Comments

Reviewer A

Comment 1: - Abstract.
Line 37. The first sentence is questionable. Serum ferritin is a good biomarker when is low, suggesting iron deficiency, but in the case of hyperferritinemia, it is not a reliable index of iron overload (see also the following sentence that contrasts the first statement).
Reply 1: Revision has been made as suggested; when it is low, it suggests iron deficiency, but in the case of hyperferritinemia it is not necessarily an index of iron overload.

Comment 2: Lines 47-48. The sentence should be modified as hyperferritinemia is not a condition of iron overload but an index of iron overload in hemochromatosis as well as in other iron overload conditions.
Reply 2: Revision has been made as suggested; On the other hand, hyperferritinemia is an index of iron overload in hemochromatosis and other iron overload conditions.

Comment 3: - Introduction.
The introduction should give the reader a general view of the issue while the Author introduces only specific points (HLH, MAS, HHCS) that do not give the reader a correct panorama of the complexity of hyperferritinemia in the clinical practice.
Reply 3: Revision has been made following the reviewer's suggestion.
The introduction has extensively been revised (see the text).

Comment 4: - Introduction.
Lines 56-57. Serum ferritin is not usually measured in case of viral or bacterial infection and inflammatory diseases because it does not give valuable information in clinical practice with few exceptions:
- MAS, where serum ferritin can be part of the diagnostic work-up
- Still's disease, juvenile acute arthritis
- Severe SARS-2 infection

Reply 4: We usually measure serum ferritin in cases of viral or bacterial infections/inflammatory diseases to evaluate the disease activity/severity. Thus, revision has been made as; Besides iron overload, high serum ferritin is caused by a non-iron overload condition, such as viral (Epstein Barr virus, SARS-CoV-2 infections, etc.) or bacterial infectious diseases (sepsis) or inflammatory diseases (Still's disease, juvenile acute arthritis, etc.), in which serum ferritin levels are employed to evaluate disease activity/severity.

Comment 5: - Iron homeostasis and ferritin.
In the paragraph, there is no description of the regulation of iron homeostasis but only

a generic sentence: “In iron metabolism, hepcidin is the main regulator while transferrin, ceruloplasmin, and ferritin also play an important role”; ok, but what about transferrin receptor, DMT1, hephestin, TFR2, HFE, HJV, TMPRSS6?
Reply 5: The author is grateful for these helpful comments. A detailed description of iron homeostasis is out of scope in this review. However, according to the reviewer’s suggestion, revision has been made as; Hyperferritinemia is largely related to disturbed iron homeostasis. In iron metabolism, many molecules are involved such as divalent metal-ion transporter 1 (DMT1), HFE, hepcidin, ferroportin 1, transferrin, ceruloplasmin, transferrin receptor, hemojuvelin, etc. of which hepcidin plays the main regulator (**1**). Iron regulatory proteins (IRPs) bind to the iron-responsive element (IRE) within the cells where iron is transferred. The IRE/IRP interaction and hepcidin are coordinated with the fluctuation of the cellular iron level (**2**).

Comment 6: Lines 86-87. The sentence is a copy and patch from the Metallomics.2021; 13: mfab021, which is taken from another controversial paper (Int J Clin Exp Pathol 2013; 6:622-629). It should be noted that ferritin molecules are heteropolymers of H- and L-ferritin and do not function as homopolymers.
Reply 6: Thank you for your criticism and for helping me revise this part. Revision has been made (see the text).

Comment 7: Lines 90-93. The author forgets the main regulatory pathway mediated by iron: Iron regulatory element (IRE) and iron-responsive proteins (IRPs)
Reply 7: Thank you for your reminder. Revision has been made commenting on the IRE/IRP interaction as shown above.

Comment 8: Lines 95-96. The glycosylation status of ferritin is a research test that has no useful utilization in clinical practice.
Reply 8: Thank you for your comments. Revision has been made by including; though the glycosylation status remains a research test and is not routinely utilized in clinical practice.

Comment 9: - Source and characteristics of ferritin
Lines 100-101. The sentence is unclear. In hemochromatosis, hepatocellular is usually mild and ferritin well correlated to liver iron overload differently from other conditions of hyperferritinemia such as chronic liver diseases, viral, alcohol-induced, metabolic.
Reply 9: Revision has been made as; In hemochromatosis and inherited iron overloading anemias associated with ineffective erythropoiesis, increased ferritin synthesis was also suggested (**13** a new reference).

Comment 10: Lines 108-109. The sentence does not consider that in HHCS the cause of hyperferritinemia is the dysregulation of the IRE/IRP system due to mutations in the IRE stem-loop. The release of ferritin from cells to blood is a consequence but not the main cause. What the Author mentions refers to the rare cases of hyperferritinemia due to mutation in the first exon of LFT. In those mentioned cases it is hypothesized but not

confirmed that the reason is an abnormal ferritin secretion. Last, there is another recently published paper reporting hyperferritinemia associated to the mutation of the STAB1 gene.

Reply 10: The author thanks for the new information on another recent hereditary hyperferritinemia report. Revision has been made as: In hemochromatosis and inherited iron overloading anemias, increased ferritin synthesis was also suggested (**13**). Among the cases of hereditary hyperferritinemia, in HHCS cases, it is due to constitutive L-ferritin production (**17**). In cases of *FTL* gene mutation cases, an abnormal ferritin secretion is hypothesized but not confirmed (**18**).In cases of STAB1 gene mutation cases, the mechanism remains to be clarified (**19**).

Comment 11: - Differentiation of hyperferritinemia with or without iron overload
Line 131. Computed tomography (CT) is not useful in defining liver iron concentration.
Line 134. Ultrasound-guided liver biopsy is not a dangerous procedure when performed by expert personnel, and this is not the reason why liver biopsy is less used than before, but only the fact that the high level of precision of T2* measurement by dedicated MRI now allows a non-invasive and efficient procedure for measurement of iron concentration in different organs.

Reply 11: Revision has been made as; liver biopsy was previously employed as the standard procedure, but is less used than before because the high level of precision of T2* measurement by dedicated MRI now allows a non-invasive and efficient procedure for measurement of iron concentration in the liver and other organs (**23,24**).

Comment 12: Table 1 A and B. The tables are redundant as the review is dedicated to hyperferritinemia in general. A table including different causes of hyperferritinemia will better serve the reader.

Reply 12: Table 1AB has been deleted. Instead, a new Table 1 showing different causes of hyperferritinemia, and Table 2 hyperferritinemia-related gene panel, have been prepared.

Comment 13: Figure 1 is incomplete and includes several inaccuracies; e.g. hereditary hyperferritinemia does not include only HHCS but also FTL- and STAB1-hyperferritinemias; the term secondary hemochromatosis is outdated as well as the term hereditary hemochromatosis (is recognized that the term hemochromatosis is enough) according to recent reviews; the Author forgets many other causes of hyperferritinemia (chronic liver diseases, hyperthyroidism, inherited and acquired iron loading anemias, aceruloplasminemia, DMT1 deficiency, …)

Reply 13: In Figure 1, HHCS has been deleted. Instead, hereditary hyperferritinemia has been employed. Hereditary and secondary hemochromatosis has been replaced by hemochromatosis and inherited iron overloading anemias. Major causes only have been included in this Figure. In the text, aceruloplasminemia, atransferritinemia, and DMT1 deficiency have been handled under a section on microcytic anemias. No detailed mention of chronic liver diseases and hyperthyroidism has been made in this review.

Comment 14: Figure 3. Is unclear why including Wilson's disease together inherited forms of iron overload. Wilson's disease is not included in the differential diagnosis as iron indices are generally normal unless hepatic damage is present. In aceruloplasminemia ceruloplasmin is below the resolution limit of the test, while in Wilson's is reduced.
Reply 14: W Wilson's disease has been deleted from the text and Figure 3.

Comment 15: - Hyperferritinemia and hemochromatosis
There is no mention of the difference between gain-of-function and loss-of-function mutations in. SLC40A1 gene, the first causing hemochromatosis type 4, the second, ferroportin deficiency that is no more included in the hemochromatosis group.
Reply 15: Revision has been made as; It has been stated as that Gene mutations of SLC40A1 encoding ferroportin, the unique cellular iron exporter, cause two types of iron overload disease; one is type 4A (ferroportin disease), which is due to "loss of function" mutations. The other is type 4B, caused by "gain of function" mutations, showing resistance to hepcidin-mediated ferroportin degradation (**88**). Currently, type 4A belongs to hemochromatosis, but type 4B is no more included in the hemochromatosis group.

Comment 16: Lines 284-285. Ceruloplasminemia and hypotransferrinemia should not be included in the paragraph as they are not forms of hemochromatosis, but different diseases.
Reply 16: Revision has been made as; they have been moved to the section of other iron overload disorders.

Comment 17: Lines 289-290. Wilson's disease and hemochromatosis are different disorders.
Reply 17: Wilson's disease has been deleted.

Comment 18: Lines 292-293. As mentioned before, the term secondary hemochromatosis is outdated. The Author should use the term iron overload, instead. Also, hemolysis does not induce relevant iron overload (in case, only in the macrophages), The cause of iron overload in some hereditary hemolytic diseases is mainly due to the associated ineffective erythropoiesis (e.g. pyruvate kinase deficiency).
Reply 18: Thank you for these important comments. Pyruvate kinase deficiency has been included in the category of iron overloading anemias. Also, regarding iron overload in DHS1, it has been mentioned that Iron overload in DSH1 (**100-102**) could be explained by chronic hemolysis, but the findings that PIEZO1 activation induced Ca^{2+} influx and suppression of HAMP expression causing low hepcidin indicate a link between PIEZO1 and iron metabolism (**94**).

Comment 19: Lines 298 – 301. The Author should explain that iron overload in CDA, sideroblastic anemias, is due to ineffective erythropoiesis inhibiting hepcidin synthesis and increasing iron absorption and release in the blood. Also, these disorders should

not be included in the paragraph hyperferritinemia and hemochromatosis.
Reply 19: Revision has been made as; These disorders have been moved to the section of other iron overload disorders, separated from hemochromatosis. It has been stated that Other inherited iron-loading anemias are characterized by ineffective erythropoiesis and hepcidin suppression (**92**). This group of anemias includes iron overload in pyruvate kinase deficiency, CDA, XLSA, and other genetic anemias. Also, comments have been made as; In these anemias, serum ferritin may be already high on non-transfusion status and become even higher after transfusions (**103**).

Reviewer B
Comment 1: The Review is easy to read contains the necessary information and remains concise. The division into three groups: non-iron overload, mixed type, and iron overload as well as the illustrations are helpful in understanding and management of the causes of hyperferritinemia.
I have a few remarks: some sentences are not quite clear to me. Rules 73/74: "There is a link between iron and copper metabolism, which is reflected in the iron overload disease." What does this mean?

Reply 1: The reason the author mentioned is that an observation of low serum ceruloplasmin in Wilson's disease is well recognized. On the other hand, ceruloplasmin, which contains greater than 95% of the copper found in plasma, is a player in iron metabolism as ferroxidase that oxidizes toxic ferrous iron (Fe2+) to its nontoxic ferric form (Fe3+). However, in the revision, a statement on Wilson's disease has been deleted in response to Reviewer 1 criticism.

Comment 2: It is stated (rule 100/101) that in hemochromatosis ferritine arises from damaged hepatic cells. Beaton and Adams, Ann of Hepatol 2012;11:294-300 see increased synthesis as the mechanism of hyperferritinemia in this condition.
Reply 2: Thank you for letting me know about Beaton and Adams's paper. Based on their report, revision has been made as; In hemochromatosis and inherited iron overloading anemias, increased ferritin synthesis was also suggested (**13**).

Comment 3: Rule 122-125: "Thus, how to define hyperferritinemia depends on the nature of the disease." A disease may result in hyperferritinemia but this does not affect the definition of hyperferritinemia.
Reply 3: Revison has been made as; Thus, as defining hyperferritinemia, strict cut-off values such as >1.5 x UNL are employed for mild ~moderate increase while a cut-off of ≥500ng/mL~ 684ng/mL could be employed for a disease showing markedly high serum ferritin.

Comment 4: Rule 237/238: "The condition is like the metabolic syndrome related to hepatic alcohol consumption – steatosis." This is too strong and not supported by the referenced literature.
Reply 4: The sentence has been deleted.

Comment 5: Rule 301-303: “Actual serum ferritin values reported in hemochromatosis were from 200 ng/mL to 2000 ng/mL in HFE-HH” The range is much wider, even up to 6000 ng/mL as is shown nicely in Figure 2. In reference 77 there is no mention of this range.
Reply 5: It is said in reference 77 that only 10% to 12% of the participants with an elevated serum ferritin level (from 200μg/L to 1000 μg/L) had the C282Y genotype. Revision has been made as 200 ng/mL to 1000 ng/mL in HFE-HH. In Figure 2, the range includes combined hemochromatosis and other iron overload anemias (non-transfusion conditions as well as post-transfusion conditions).

Reviewer C
Comment 1: How do serum ferritin levels differ between metabolic hyperferritinemia and rare hereditary conditions like HHCS?
Reply 1: Serum ferritin levels don’t differ; however, the glycosylated status of ferritin may differ (However, an assay of the glycosylated status is not routinely available). Also, target gene sequencing for hereditary hyperferritinemia and other inherited iron overload disorders is essential. These problems have been discussed as future perspective.

Comment 2: Why might middle-aged adults with mild to moderate hyperferritinemia be referred to hematologists in the absence of infectious or inflammatory diseases?
Reply 2: The sentence was unclear. Revision has been made as; It is not infrequent that middle-aged adult patients with mild to moderately elevated serum ferritin (500ng/mL~3,000ng/mL) are referred to hematologists as unknown hyperferritinemia. Physicians need to be aware of metabolic hyperferritinemia with or without iron overload.

Comment 3: How can imaging studies be useful in the differentiation of hyperferritinemia conditions?
Reply 3: Particularly, an MRI study of the liver is useful for assuming the liver iron content (LIC), which may add additional information if iron overload is present or not. (These have been discussed in Lines 145-148 of the revised manuscript.)

Comment 4: Can hyperferritinemia be a standalone diagnostic marker, or is it typically assessed in conjunction with other tests?
Reply 4: Hyperferritinemia may not be a standalone, specific diagnostic marker. We can tell from serum ferritin levels to assess the disease activity when patients show infectious or inflammatory diseases or suspicious iron overload conditions. However, we have to be careful in the differential diagnosis of the cause(s) of high serum ferritin levels, because in some cases, the cause(s) of hyperferritinemia can’t be explained. For such cases, target gene sequencing may be necessary. These problems have been discussed as future perspectives.

Comment 5: What challenges do clinicians face in differentiating between various causes of hyperferritinemia?
Reply 5: It is particularly challenging if the patients have hemochromatosis or other inherited iron-loading anemias. We often require target sequencing to reach a correct diagnosis. Therefore, in this revised version, Table 2 (hyperferritinemia-related gene panel) and comments have been made in the future perspectives.

Comment 6: How might new research on ferritin and iron metabolism impact the future diagnosis and treatment of hyperferritinemia?
Reply 6: From a future perspective, assays of hepcidin and glycosylated ferritin status should be available in daily practice. In addition, target gene sequencing analysis is encouraged to be applied broadly for inherited iron metabolism abnormalities and iron-loading anemias using a gene panel in Table 2.

Comment 7: Concerning future perspective: can you speculate a little about
1 new player as Calprotectin, Omentin? or other players
2 How do you imagine the future or perspective? Any role for Artificial Intelligence?

1. **Reply 7**: Calprotectin is known to chelate iron and deprive bacteria of this essential nutrient, which may affect iron homeostasis. Omentin-1 (one of the adipokines) is involved in insulin resistance, and serum inflammatory markers in obese subjects with metabolic syndrome, which may be related to metabolic hyperferritinemia. However, in this review, it seems too early to speculate the involvement of these new molecules in hyperferritinemia.
2. The application of artificial intelligence (AI) in medicine is an important topic. Of course, the future potential of AI in the differential diagnosis of hyperferritinemia may exist and be interesting. However, for this review, the author is afraid to speculate without AI-applied base studies in this field.