BMJ Open publishes all reviews undertaken for accepted manuscripts. Reviewers are asked to complete a checklist review form ([http://bmjopen.bmj.com/site/about/resources/checklist.pdf](http://bmjopen.bmj.com/site/about/resources/checklist.pdf)) and are provided with free text boxes to elaborate on their assessment. These free text comments are reproduced below.

### ARTICLE DETAILS

<table>
<thead>
<tr>
<th>TITLE (PROVISIONAL)</th>
<th>HFE GENE MUTATION AND OXIDATIVE DAMAGE BIOMARKERS IN PATIENTS WITH MYELODYSPLASTIC SYNDROMES AND ITS RELATION TO TRANSFUSIONAL IRON OVERLOAD - OBSERVATIONAL CROSS-SECTIONAL STUDY</th>
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<tbody>
<tr>
<td>AUTHORS</td>
<td>de Souza, Geane; Ribeiro-Jr, Howard; Sousa, Juliana; Heredia, Fabiola; Freitas, Rivellison; Martins, Manoel; Gonçalves, Romélia; Pinheiro, Ronald; Magalhães, Silvia Maria</td>
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### VERSION 1 - REVIEW

| REVIEWER             | Zhijian Xiao  
|----------------------|---------------------------------------------------------------|
|                      | MDS Centre  
|                      | Blood Diseases Hospital & Institute of Hematology  
|                      | Chinese Academy of Medical Science & Peking Union Medical Sciences  
|                      | China |
| REVIEW RETURNED      | 20-Jul-2014 |
| GENERAL COMMENTS     | "OXIDATIVE DAMAGE" in the title should be "OXIDATIVE DAMAGE Biomarkers".  
|                      | Major comments:  
|                      | 1. The total number of RBC transfusion should be mentioned in the manuscript;  
|                      | 2. The LPI of the patients were measured? |

| REVIEWER             | Norbert Gattermann  
<table>
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<td>Heinrich-Heine-University Düsseldorf, Germany</td>
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<tr>
<td>REVIEW RETURNED</td>
<td>02-Aug-2014</td>
</tr>
<tr>
<td>GENERAL COMMENTS</td>
<td>This group of authors, who last year published a paper on &quot;Increased parameters of oxidative stress and its relation to transfusion iron overload in patients with myelodysplastic syndromes&quot; in J Clin Pathol, have now tried to find a correlation between HFE gene mutation and oxidative damage in MDS patients. HFE mutations (mostly heterozygous H63D) were identified in 24 of 78 (30.8%) of patients and 5 of 87 (5.7%) of volunteers. Only one patient showed a constellation that is known to cause hereditary hemochromatosis, namely double heterozygous C282Y/H63D. Nevertheless, having an HFE mutation was observed to be significantly associated with higher serum ferritin levels in MDS patients with iron overload as well as MDS patients without IOL. Markers of oxidative stress were significantly correlated with iron overload (SF &gt;1000 ng/ml) but not with HFE gene mutations.</td>
</tr>
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</table>
Questions/comments:

In view of the current controversy surrounding iron chelation therapy in transfusion-dependent MDS patients, it is somewhat overstated to say that "excess of iron, due to multiple transfusions, is extremely toxic ..." (introduction, p. 3). The authors should leave out the exaggerating adverb.

Could the authors please explain why they refer to HFE gene variants sometimes as mutations and sometimes as polymorphisms?

Do the authors have an idea why HFE gene variants were significantly more frequent in MDS patients (with and without IOL) than in normal controls?

Can the authors explain why HFE mutation was significantly associated with higher serum ferritin levels, whereas no significant difference in the frequency of HFE gene mutation was found between MDS patients with and without IOL?

In their discussion, the authors say that "... MDA values were significantly higher in patients with IOL when compared to patients without IOL and this was independent of the polymorphism in the HFE gene, ..." whereas in the results section, they say that "... in iron overloaded patients ... the values of MDA were significantly higher in patients with HFE gene mutation". Could the authors please resolve that discrepancy?

In the discussion, the authors may wish to refer to some of the following publications also dealing with HFE mutations in MDS patients:

HFE genotype and iron metabolism in Chinese patients with myelodysplastic syndromes and aplastic anemia.

Hemochromatosis gene mutation—could it be a disease marker for myelodysplasia?

Hemochromatosis-associated gene mutations in patients with myelodysplastic syndromes with refractory anemia with ringed sideroblasts.

The significance of the hemochromatosis genetic variants in multiple myeloma in comparison to that of myelodysplastic syndrome.

Prevalence of hemochromatosis gene (HFE) mutations in Greek patients with myelodysplastic syndromes.
This paper will require very substantial improvement in order to merit publication.

Multivariate analysis is needed but has not been performed.

Oxidative stress has profound effects on cellular biology, affecting mitochondrial function, membrane integrity, and activity of signalling pathways as well as promoting DNA damage and carcinogenesis. This has become a hot topic in MDS research, particularly in the context of secondary haemochromatosis related to RBC transfusion.

In this paper the authors measure a marker of oxidative damage of lipid membranes (plasma malonaldehyde) and two components of the antioxidant defense system (glutathione peroxidase and superoxide dismutase) in MDS patients and normal controls. They find that MDA, GPx, and SOD are present in higher concentrations in iron-overloaded patients than in non-overloaded patients or controls. In their dataset, presence of an HFE mutation is associated with higher serum ferritin, but not with increase MDA, GPx, or SOD in the absence of iron overload. The main conclusion of the paper is that "neither high concentration of oxidant agents nor the increase in antioxidant capacity showed significant relation to HFE gene mutation in patients with MDS without IOL, showing that mutation did not account for oxidative stress".

The work described in this paper is for the most part appropriately conducted and analysed, and will be of some interest to workers in this field. However, there are several issues that prevent me recommending publication in its current form.

1. The rationale underpinning the main hypothesis – that HFE mutation will have an impact independent of iron overload on oxidative stress in MDS – is not articulated in the manuscript.
Indeed, it is not clear why one might imagine this could be the case. HFE regulates iron absorption via its interactions with hepcidin and the transferrin receptor, and so one may easily imagine an effect on oxidative stress mediated via iron and the Fenton reaction. Do the authors propose another potential mechanism through which HFE mutation might affect oxidative stress? If so, it needs exposition in the manuscript. If not, the main hypothesis and main conclusions of the paper seem not to make sense.

2. The novelty of the study is otherwise very limited. It is known that IOL increases oxidative stress in MDS (as cited by the authors of this manuscript).

3. Given the main conclusion of the work as stated in the abstract, it is surprising that the authors have not performed a proper multivariate analysis to dissect the impacts of iron overload and HFE mutation.

4. With a patient group of this size it may have been possible for the authors to seek correlations between oxidative stress parameters and clinical outcome. It is unfortunate that no such analysis is presented.

5. The description of the patients is insufficiently detailed. No information is offered on given regarding treatments received; most glaringly, no information is given on iron chelation therapy. Chelation therapy has a rapid effect on the presence of “free” iron, such that physiological control of this mediator of oxidative stress occurs long before iron stores (as estimated by serum ferritin measurement) are reduced. If some or all of the patients in this study were receiving ICT the validity of the conclusions will be greatly reduced.

6. Although the paper is generally quite well written there are numerous small errors in grammar and spelling. Careful proofreading is recommended.

**VERSION 1 – AUTHOR RESPONSE**

Reviewer: 1

Questions/comments:

1- "OXIDATIVE DAMAGE" in the title should be "OXIDATIVE DAMAGE biomarkers".

Included in the title.

Answer: HFE gene mutation and oxidative damage biomarkers in patients with myelodysplastic syndromes and its relation to transfusional iron overload.

2- The total number of RBC transfusion should be mentioned in the manuscript:

Included in Results.

Answer: The patients with IOL received a mean of 42.8 units of RBC and had a mean serum ferritin level of 2,880 ng/mL.

3- The LPI of the patients were measured?

Answer: The determination of the LPI was not performed in this study, but is planned to be performed in future studies by our group.
Reviewer: 2
Questions/comments:

1. In view of the current controversy surrounding iron chelation therapy in transfusion-dependent MDS patients, it is somewhat overstated to say that “excess of iron, due to multiple transfusions, is extremely toxic ...” (introduction, p. 3). The authors should leave out the exaggerating adverb.

Answer: Excess of iron, due to multiple transfusions is toxic through the production of free radicals derived from activated oxygen species, which eventually form hydroxyl radicals from superoxide or hydrogen peroxide resulting in organ dysfunction.

2. Could the authors please explain why they refer to HFE gene variants sometimes as mutations and sometimes as polymorphisms?

“Variation of the HFE gene” was standardized in the text

Answer: Polymorphism and mutation can be defined as alternative or variant form of a gene that occupies a specific location on a chromosome (MOHRENWEISER; WILSON and JONES, 2003). Polymorphism is genetic disorder that occurs in more than 1% of the population (TAKASHIBA; NARUISHI, 2006). Mutations are rare (occurs < 1% of the population). In the HFE gene an average of 20 mutations were identified: The C282Y and S65C mutations are rare while H63D has a frequency above 1%. Variation of the HFE gene was standardized in the text according to reviewer suggestion.

3- Do the authors have an idea why HFE gene variants were significantly more frequent in MDS patients (with and without IOL) than in normal controls?

Answer: HFE variants correlating with body iron levels have shown association with cancer risk (Beckman LE et al, 1999), including childhood acute lymphoblastic leukemia (Kennedy AE et al, 2014), mediated mainly via their effect on body iron levels or linkage disequilibrium with nearby polymorphisms. The incidence of HFE mutations in patients with MDS remains controversial. Increased prevalence of HFE mutations is not a generalized feature of MDS although some subgroups exhibit mutations at a higher frequency than others and then healthy controls (Nearman ZP et al, 2007). One limitation of this study could have been the effect modification by gender, as most healthy controls were female (90%), considering that previous associations of HFE variants with gender effect were reported. Finally, an ethnicity-specific difference could also play a role as these mutations were present in 50% of Hungarian myelodysplastic cases significantly higher than incidence found in controls (Várkony J et al, 2009). Secondary analyses with an increased number of patients are necessary for ultimate confirmation.

4- Can the authors explain why HFE mutation was significantly associated with higher serum ferritin levels, whereas no significant difference in the frequency of HFE gene mutation was found between MDS patients with and without IOL?

Answer: In the specific group of MDS patients the increased absorption of iron secondary to decrease in hepcidin level and the transfusional iron loading were the main factors of iron overload, probably overcoming the effect of the presence of HFE variant.

5- In their discussion, the authors say that “… MDA values were significantly higher in patients with IOL when compared to patients without IOL and this was independent of the polymorphism in the HFE gene …” whereas in the results section, they say that “… in iron overloaded patients … the
values of MDA were significantly higher in patients with HFE gene mutation”. Could the authors please resolve that discrepancy?

Answer:
Discussion: MDA values were significantly higher in patients with IOL when compared to patients without IOL and this was supposed to be independent of the polymorphism in the HFE gene, once the presence of HFE variant did not show the same effect in patients with no IOL. The presence of mutation in the group without IOL did not affect directly the parameters of the lipid peroxidation.

Results: Levels of MDA were higher in the IOL group compared to the group without IOL. When MDA levels were compared according to the presence of the HFE gene variant for patients without IOL no statistically significant difference was observed, although there was a trend of increase in the concentration of MDA in the group with HFE variant.

Reviewer: 3
Questions/comments:

1- The rationale underpinning the main hypothesis – that HFE mutation will have an impact independent of iron overload on oxidative stress in MDS – is not articulated in the manuscript. Indeed, it is not clear why one might imagine this could be the case. HFE regulates iron absorption via its interactions with hepcidin and the transferrin receptor, and so one may easily imagine an effect on oxidative stress mediated via iron and the Fenton reaction. Do the authors propose another potential mechanism through which HFE mutation might affect oxidative stress? If so, it needs exposition in the manuscript. If not, the main hypothesis and main conclusions of the paper seem not to make sense.

Answer: As in clinical practice, some patients present with more relevant increase in ferritin levels than others, having received the same transfusional iron loading, we hypothesized the presence of HFE gene variant would contribute to this phenomenon and to a more severe increase in oxidative biomarkers. Moreover, the incidence of HFE mutations in patients with MDS remains controversial and has been found to be increased in some MDS population in previous reports. These observations were added to the discussion section.

2- The novelty of the study is otherwise very limited. It is known that IOL increases oxidative stress in MDS (as cited by the authors of this manuscript).

Answer: The incidence of HFE mutations in patients with MDS remains controversial and has been found to be increased in some MDS population in previous reports. If the presence of HFE gene variant contributes to a more expressive increase in ferritin level and oxidative stress biomarkers still a matter of debate. Moreover HFE variants correlating with body iron levels have shown association with cancer risk (Beckman LE et al, 1999), including childhood acute lymphoblastic leukemia (Kennedy AE et al, 2014), mediated mainly via their effect on body iron levels or linkage disequilibrium with nearby polymorphisms.

3- Given the main conclusion of the work as stated in the abstract, it is surprising that the authors have not performed a proper multivariate analysis to dissect the impacts of iron overload and HFE mutation.

Answer: A multivariate analysis will certainly be performed as an increased number of patients are included. The authors thank the reviewer for the suggestion.

4- With a patient group of this size it may have been possible for the authors to seek correlations between oxidative stress parameters and clinical outcome. It is unfortunate that no such analysis is
presented.

Answer: The proposal was to perform an observational, cross-sectional analysis. The idea of seeking correlation between oxidative stress biomarkers and clinical outcome could be the aim of the following analysis. The authors thank the reviewer for the suggestion.

5- The description of the patients is insufficiently detailed. No information is offered regarding treatments received; most glaringly, no information is given on iron chelation therapy. Chelation therapy has a rapid effect on the presence of “free” iron, such that physiological control of this mediator of oxidative stress occurs long before iron stores (as estimated by serum ferritin measurement) are reduced. If some or all of the patients in this study were receiving ICT the validity of the conclusions will be greatly reduced.

Answer: All the patients were included at diagnosis and all the samples were collected prior to any treatment or chelator therapy. These observations were added to the Material and Methods section.

6- Although the paper is generally quite well written there are numerous small errors in grammar and spelling. Careful proofreading is recommended.

Answer: Careful revision was performed

<table>
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<tr>
<th>REVIEWER</th>
<th>Zhijian Xiao</th>
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<tr>
<td>Institute of Hematology and Blood Diseases Hospital, Chinese Academy of Medical Sciences &amp; Peking Union Medical College, China</td>
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| REVIEW RETURNED | 19-Oct-2014 |

- The reviewer completed the checklist but made no further comments.

<table>
<thead>
<tr>
<th>REVIEWER</th>
<th>Richard A. Wells</th>
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| Sunnybrook Health Science Centre  
University of Toronto  
Canada |

| REVIEW RETURNED | 15-Oct-2014 |

| GENERAL COMMENTS | After the incorporation of revision by the authors the paper is improved. However, I have the following residual concerns:  
1. It is stated that the patients in this study were enrolled at the time of diagnosis and analyses were performed prior to any therapy being instituted. However, in the IOL group an average of over 40 blood transfusions had been received. This implies that these patients had transfusion-dependent anaemia for a period of years prior to their enrolment in the study. Were they all undiagnosed during this pre-study period, and were no treatments other than regular blood transfusion given to any of the patients? Such a pattern of care would be unusual in many parts of the world and as such deserves an explanatory comment in the text.  
2. In the discussion it is asserted: “It would be reasonable to hypothesize that the presence of HFE gene variation contributes to increased serum MDA values.” It remains unclear how this could be, aside from via iron overload. Could the authors clarify explicitly how HFE gene variation might affect oxidative stress independently of |
iron status? In their response the authors suggest that patients with HFE variants might develop iron overload more rapidly than those with lid-type HFE. This may indeed be the case – can the authors address the question with their data, for example by correlating serum ferritin with number of red cell units transfused in HFE wild type vs HFE variant patients?

3. I note that the data in this publication overlap with data previously published by this group de Sousa et al., J Clin Pathol Nov 2013 Vol 66 No 11 pp 996-998). In particular the serum ferritin and MDA results are included in this publication. This serves further to limit the novelty of the results presented in the current manuscript. The authors should at least acknowledge explicitly in the text that this group of patients and their serum ferritin and MDA values have been reported previously and are not part of the data presented here.

4. In Results (page 6, final paragraph) it is stated that “seven patients with IOL were identified with mutation in the HFE gene”. However, in Table 2 it appears 8 patients are listed. I think one patient is counted twice. This should be corrected.

**VERSION 2 – AUTHOR RESPONSE**

Reviewer: 2
Questions/comments:

4- Can the authors explain why HFE mutation was significantly associated with higher serum ferritin levels, whereas no significant difference in the frequency of HFE gene mutation was found between MDS patients with and without IOL?

Answer: Discussion: It would be reasonable to hypothesize that the presence of HFE gene variation contributes to increased serum MDA values. However, Parkkila et al demonstrated that HFE gene variation did not account for additional harm in transfusional iron overloaded patients with acute myeloid leukemia29. In the present report statistical analysis showed no significant difference when patients with and without gene variation were compared. In the specific group of MDS patients the increased absorption of iron secondary to decrease in hepcidin level and the transfusional iron loading were the main factors of iron overload, probably overcoming the effect of the presence of HFE variant.

5- In their discussion, the authors say that “... MDA values were significantly higher in patients with IOL when compared to patients without IOL and this was independent of the polymorphism in the HFE gene ...” whereas in the results section, they say that “... in iron overloaded patients ... the values of MDA were significantly higher in patients with HFE gene mutation”. Could the authors please resolve that discrepancy?

Answer: Discussion: In this study, MDA values were significantly higher in patients with IOL when compared to patients without IOL and this was supposed to be independent of the polymorphism in the HFE gene, once the presence of HFE variant did not show the same effect in patients with no IOL. The presence of mutation in the group without IOL did not affect directly the parameters of the lipid peroxidation.

Results: Iron overloaded patients showed a significant increase in plasma MDA when compared with all groups (p<0.0001). Levels of MDA were higher in the IOL group compared to the group without IOL. When MDA levels were compared according to the presence of the HFE gene variant for patients without IOL no statistically significant difference was observed, although there was a trend of increase in the concentration of MDA in the group with HFE variant (p=0.2789) (Table 3).
Reviewer: 3

Questions/comments:

1- It is stated that the patients in this study were enrolled at the time of diagnosis and analyses were performed prior to any therapy being instituted. However, in the IOL group an average of over 40 blood transfusions had been received. This implies that these patients had transfusion-dependent anaemia for a period of years prior to their enrolment in the study. Were they all undiagnosed during this pre-study period, and were no treatments other than regular blood transfusion given to any of the patients? Such a pattern of care would be unusual in many parts of the world and as such deserves an explanatory comment in the text.

Answer: In the IOL group, we detected an average of over 40 blood transfusions. This number is high but the Brazil is a poor country with 180 million people and all the health care system is provided by the government. Unfortunately, the patients wait for up to one year before getting an interview with the hematologist. This may explain the high number these patients with increased serum ferritin at diagnosis.

2- In the discussion it is asserted: “It would be reasonable to hypothesize that the presence of HFE gene variation contributes to increased serum MDA values.” It remains unclear how this could be, aside from via iron overload. Could the authors clarify explicitly how HFE gene variation might affect oxidative stress independently of iron status? In their response the authors suggest that patients with HFE variants might develop iron overload more rapidly than those with lid-type HFE. This may indeed be the case – can the authors address the question with their data, for example by correlating serum ferritin with number of red cell units transfused in HFE wild type vs HFE variant patients?

Answer: The HFE is a protein responsible for the internalization of iron in enterocytes and the C282Y variant is more involved in this process. Among the cases without IOL, we detected H63D variant in 17 patients. Among the cases with IOL only one related to C282Y. This difference was not significant probably due to the low number of cases in each group. The C282Y mutation which is more commonly associated with iron overload was detected in only one patient. Correlate serum ferritin with the number of red cell units transfused certainly will be performed as increased number patients. The authors thank the reviewer for the suggestion.

3- I note that the data in this publication overlap with data previously published by this group de Sousa et al., pp 996-998). In particular the serum ferritin and MDA results are included in this publication. This serves further to limit the novelty of the results presented in the current manuscript. The authors should at least acknowledge explicitly in the text that this group of patients and their serum ferritin and MDA values have been reported previously and are not part of the data presented here.

Answer: 101 adult patients with MDS seen at University Hospital of the Federal University of Ceará, Brazil, between May, 2010 and September, 2011 participated in the survey and were analyzed iron status, oxidative stress, genetic variant and cytokines. Preliminary results by some individuals been mentioned in previous published study. The control group was composed of 87 healthy volunteers (preliminary results by 45 individuals healthy also been mentioned in J Clin Pathol Nov 2013 Vol 66 No 11).

4. In Results (page 6, final paragraph) it is stated that “seven patients with IOL were identified with mutation in the HFE gene”. However, in Table 2 it appears 8 patients are listed. I think one patient is counted twice. This should be corrected.

Answer: One patient had double heterozygous (C282Y/H63D), results shown as.
Table 2: Legend:
*This patient had double heterozygous (C282Y/H63D).

Reviewer: 1
Please state any competing interests or state ‘None declared’: None declared.
Conflict of Interest:
The authors declare there are no competing interests or state.

VERSION 3 - REVIEW

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<th>REVIEWER</th>
<th>Richard Wells</th>
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<td>University of Toronto</td>
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<td>Canada</td>
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<td>REVIEW RETURNED</td>
<td>25-Nov-2014</td>
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GENERAL COMMENTS

The manuscript now contains the new statement: "We hypothesize if the presence of HH gene variations, in both homozygous and heterozygous forms, could contribute to a significantly higher rate of iron accumulation in the context of transfusion dependence." This is excellent. However, the authors do not test this hypothesis. I request that they do so -- please simply compare the relationship between units transfused and SF in HFE variant carriers vs non-variants. In their letter of response the authors promise to do this in a later study. Why not now? All the necessary data are at hand -- SF and number of transfusions at the time of diagnosis. This result would at least address their hypothesis.

VERSION 3 – AUTHOR RESPONSE

When analyzing the number of MDS patients with mutations in HFE gene was verified that 95.7% had received more than 20 transfusions, suggesting that the presence of mutations in HFE gene can be directly correlated with the need for blood transfusion, which can induce to iron overload. The results of this study suggested that the presence of HFE gene variations, in both homozygous and heterozygous forms, can be directly correlated with the need for blood transfusion, which can induce to iron overload. There was a direct relationship between oxidative stress markers and iron overload, but no was observed significant effect with the HFE gene variation and the oxidative stress markers in patients with MDS.
HFE gene mutation and oxidative damage biomarkers in patients with myelodysplastic syndromes and its relation to transfusional iron overload: an observational cross-sectional study

Geane Felix De Souza, Howard Lopes Ribeiro, Jr, Juliana Cordeiro De Sousa, Fabíola Fernandes Heredia, Rivelilson Mendes De Freitas, Manoel Ricardo Alves Martins, Romélia Pinheiro Gonçalves, Ronald Feitosa Pinheiro and Silvia Maria Meira Magalhães

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