Author’s response to reviews

Title: Protocol and baseline data for a Prospective open-label explorative randomized single centre comparative study to determine the effects of various intravenous iron preparations on markers of oxidative stress and kidney injury in chronic kidney disease (CKD). (IRON-CKD)

Authors:

Ahmed Zeidan (mda05az@doctors.org.uk)

Sunil Bhandari (Sunil.Bhandari@hey.nhs.uk)

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Editor

TRIALS

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Thank you for your interest in our manuscript

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Prospective open-label explorative randomized single-centre comparative study to determine the effects of various intravenous iron preparations on markers of oxidative stress and kidney injury in chronic kidney disease (CKD). (IRON-CKD)

Ahmed Zeidan, BSc (Hons), MBChB; Sunil Bhandari, MBChB, FRCP, PhD, MEd

We thank you very much for your kind invitation to submit a revised version of our paper. We certainly appreciate the comments of the three reviewers, which were very valuable and which helped us in substantially improving our manuscript. We have taken good care in addressing the reviewers’ concerns and have addressed them as follows:

Reviewer #1: In this manuscript, the authors propose a prospective open-label explorative randomized single-center comparative study to determine the effects of various intravenous iron
preparation on markers of oxidative stress, inflammation and kidney injury in chronic kidney disease. Secondary outcomes include examining the haematic profiles and haemoglobin concentration, as well as, arterial stiffness and "quality of life".

It is feasible that the outcome of the study could inform on future clinical management for the patient cohort described herein. Moreover, the secondary outcome could help clarify the clinical implications of iron metabolism ultimately improving patient quality of life. However, there are several major and minor revision that should be considered.

Major Revisions:

1) The sample size is very small. Given the variability of F2-Isoprostanes and MDA measured by immunoassays in healthy population (see meta-analysis: Redox Biology (2017), 12, 582-599), do the authors anticipate that 40 patients will be sufficient to achieve statistical significance in their study? If they are using descriptive statistics, what sort of conclusions are the authors expecting to draw if variation is high and F2-IsoPs and MDA readings do not correlate? Perhaps a larger number needs to be recruited to make the study more impactful. It is also unclear whether the authors will use urine or plasma to measure F2-IsoPs.

We agree that the study is small and is explorative in nature to allow for a power calculation for a more substantive study if there is the suggestion of a possible signal. We have a robust measure of serum for oxidative stress markers rather than urine due to the more instability of urine samples but recognise that urine can be used if processed promptly. However, we acknowledge that measurement can be variable and affected by a number of variables which we have attempted to limit as far as possible. Moreover, factors like acute illness during the study or having additional chronic inflammatory conditions like rheumatoid arthritis can increase plasma markers F2-Isops and MDA at variable levels. Such factors are documented clearly in patients notes and will be looked at more closely when we analyse the complete data set for the 40 patients.

2) The race of the patients is unclear. At least one study has found a significant correlation between MDA levels and race, as well as sex (see: American Journal of Epidemiology (2002), 156(3), p274-285). How will the authors control for these cofounding factors?
The majority of patients are white British as the geography of the region is composed of 92% white and we felt that the cost of patient information leaflets in other languages would make the study more difficult to deliver in the financial envelope. The ethics committee was aware of this limitation and approved the study. Regarding gender we will analyse the data stratified for gender but again as numbers are small the interpretation may be limited for the whole data-set. We had added text to indicate this in the revised manuscript.

3) Diabetes has shown in previous studies involving hemodialysis patients to have a higher propensity for lipid peroxidation during parenteral iron infusion. More than a quarter of the patients are diagnosed with DM. This can be a confounding element or independent risk factor for inflammation. How will the authors address this (and other) confounding elements?

Again because of the size of this explorative study we can examine the impact of diabetes (n=13) in the whole cohort versus non diabetes (n=27) but this analysis will be simply hypothesis generating rather than conclusive. Again we have added text to this effect. We are also aware that many of our participants are on lipid lowering medication such as statins. This can also be considered a factor that has an on lipids peroxidation and activities of antioxidants enzymes in patients with dyslipidemia. Broncel et al. have shown that in patients with dyslipidemia without clinical symptoms of atherosclerosis atorvastatin, simvastatin and pravastatin decreased similar TBARS concentrations in the isolated erythrocyte membranes. It was observed a significant increase of the antioxidant enzyme activities during atorvastatin and simvastatin treatment. This will be taken into consideration as far as possible.


4) Only one of the newer, third generation iron formulations is evaluated in the study. These newer iron formulations have been determined to be safer and with less acute side effects. Given the advantages of the newer formulations, the authors need should clarify why they have chosen to evaluate only one of these formulations. Is it related to cost? Availability?

We agree with the reviewer this is a limitation given the 3rd generation irons are safer. As suggested we only studied those irons available in our Hospital. The only other preparation
available in the UK is iron carboxymaltose which was studied as it was not available to us because of cost.

Minor Revisions:

1) The etiology of anemia specifically Iron deficiency anemia (IDA) is in some cases attributed to malnutrition. As such nutritional status should be included in the baseline characteristics in the form of serum albumin or pre-albumin.

We have added albumin to the baseline data as a separate line but it is part of the BCP which we had not clarified in the original manuscript. We recognise this may be influenced by inflammation but it also seems to correlate with levels of Hb, SF, TSAT and even eGFR

2) Evaluation of the immediate consequence of hypophosphatemia maybe considered for each infusion group.

We monitor patients closely for adverse effects and any events will be recorded. However given the literature we do not expect episodes of severe hypophosphataemia. Dr. Zeidan will be in touch with all participants who complete the study for at least a year. Any patient identified to be at risk will be immediately discussed with the caring consultant and if required reviewed in outpatient clinic.

3) Which inflammatory markers will be examined? It is good that the authors mentioned that NGAL is also increased during inflammation. But it is unclear how will the authors distinguish between NGAL caused by inflammation and related to acute kidney injury in CKD patients which may have low chronic inflammation. Also recent studies has shown in increase in NGAL levels associated with iron stores in CKD patients with anemia.

We agree that this differentiation will be difficult but we have measured CRP as a measure of inflammation and white cell count. Other inflammatory markers which are being analysed at the university lab include the Cytokines - IL-6; IL-8 and IL-10
4) Line 144 - no reference for the "specific levels" of the NGAL that are related to inflammation versus AKI.

We have added a reference in the revised paper

We have adjusted the text to indicate that trials have demonstrated that a plasma NGAL (at a cut-off value of 50 μg/L) were powerful independent predictors of AKI, with an area under the receiver-operating characteristic curve (AUC) of 0.91[22]


5) It appears that majority of patients fall in the CKD stage IV where they may be at higher risk of developing AKI, given that CKD itself is a state of low chronic inflammation. As the authors hope to determine the impact of IV iron solutions on markers of AKI this patient cohort may present a problem.

We thank the authors for their comments but as the patients chosen will be stable, any impact on renal function will most likely be related to the iron unless there is a concomitant acute event which will be noted and recorded during the analysis. Since the study is an open label, we will have had access to results from all follow up visits.

6) Drugs that patient use including Proton pump inhibitors should be mentioned.

We will ensure medication history is recorded as detailed in Table 2 at each visit and examined in the analysis but again with the small numbers any sub- analysis will be difficult to interpret.

Reviewer #2: The Authors of the IRON-CKD study have endorsed the protocol in a clear and interesting presentation.

I have the following minor suggestions
1) In the Introduction section page 6, the paragraph "When IV Iron is administrated it passes to the reticulo-endothelial system (RES). The Iron complex with either dextran or sucrose splits……...". This paragraph does not mention anything about the pathophysiological effect of Isomaltoside. Actually nowhere in the introduction I could find any explanation of its role in accelerating oxidative inflammation. Please explain as this is an important part of the rationale of the study.

We have revised the introduction to include a statement as detailed below and including a reference about the effect of isomaltoside administration and splitting from its iron in a similar but much slower fashion and hence the impact on oxidative stress is thought to be reduced.

"Iron isomaltoside consists of a linear and unbranched oligosaccharide carbohydrate moiety where the iron is tightly bound in a matrix structure. This enables a controlled and slow release of iron to iron-binding proteins and passage to the RES thus avoiding potential toxicity from release of labile iron. The strongly bound iron within the iron isomaltoside formulation allows flexible dosing, over a short time period. Compared to compounds in which iron is more loosely bound in the complex, the iron isomaltoside complex potentially leads to generation of less oxidative stress and less immunological toxicity"

2) Please explain if patient were categorized according to etiology of CKD prior to randomization, as patients with diabetic nephropathy have been found to have higher levels of NGAL. If not, then how this bias will be addressed in the analysis?

There was no stratification to aetiology of CKD but we realise the potential impact of diabetes to markers of oxidative stress and NGAL. We will carry out a post hoc comparison in the whole group to see if there is a signal and add this to the findings but realise this is hypothesis generating only and with only 13 diabetics will be of limited value.

3) Please provide SPIRIT checklist as a supplementary file.

A SPIRIT check list is attached to the revised paper as a separate file to ensure that where needed the relevant areas have been included.
4) Please describe clearly the primary and secondary outcomes of the study. The title of the study implies that the level of the biomarkers would be the primary outcome.

The review is correct that in the explorative study the primary outcomes are biomarkers but we recognise this is a small study and may not lead to definitive data but may allow for a future power calculation in a larger study. We have now clearly labelled primary and secondary outcomes but in reality it is somewhat mechanistic in nature.

5) Please state clearly in the title that this is a study protocol.

The title has been revised to reflect the above.

6) Please explain whether the markers of inflammation will be measured as a single measurement or serially.

The measurements are serial at each visit attended for each patient.

7) The results section is not necessarily a part of the study protocol. Please consider trimming down this section.

We have revised the results section to focus mainly on the planned analysis however the paper is a combination of the protocol and baseline data as stated in the title.

Reviewer #3: This manuscript describes the protocol for a 4-group randomized pilot trial of IV iron preparations for patients with CKD, and some preliminary results on the trial population's accrual and baseline characteristics. The manuscript is decently written (with a few English language errors) and thus fairly clear and easy to read. The list and details of outcomes need to be cleaned up. I have no.
The Primary and secondary and other outcomes of this explorative study are now detailed more clearly.

Major Essential Revisions - None

Minor Essential Revisions

1. There a number of English language errors mostly beginning at line 225 in the randomization procedure section but continuing until the end of the manuscript. Most of the manuscript is written in the past tense but the tense changes in a few places.

   The manuscript has been revised to remove errors and adjust tense.

2. While pilot trials are explorative, I am not sure it is accepted practice to use the term when describing the design. It might be better to use the term pilot throughout and then in the objectives and the sample size section emphasize that pilot trials are explorative and so ...

   Many thanks for this suggestion. Based on the international recommendations this method of description is the correct terminology as opposed to a feasibility study. We have adjusted the revised manuscript.

3. In the Abstract Results, the mean age has an error.

   Corrected with thanks.

4. Iron needs to be inserted after IV in line 90.

   Corrected with thanks.
5. The outcomes are described before and after the Iron administration section. The timing of each outcome is laid out in Table 2 and can simply be referenced. The outcomes should be fully described under one section, detailed thoroughly and organized better. Results and timing should not be mixed up with the outcome section.

   a. What specifically is included in the biochemical profile and the full blood count?

   b. What measures are included in oxidative stress and what further measures of inflammation will be considered beyond CRP?

   c. How was temperature measured?

   d. How was blood pressure measured?

   e. Are there specific minor adverse events that are expected?

We have revised the manuscript to reflect this. We have expanded the details to reflect comments a to e. specifically we have added the biochemical measures recorded; The inflammatory markers include urinary ACR or PCR. We may have the possibility of measuring IL-2 IL-6 and IL-10 but these will depend on funding and sample availability; details of measured of observations which is standard using an ear sensor and automated BP cuff. Minor events may include tachycardia and flushing as in the Fishbane reaction and arthralgia as the main reactions.

6. In Line 226, were these blocks permuted?

   No – the order was not adjusted during the study

7. The results in lines 257-259 should be moved to the Result section.

   These have been moved and reduced to accommodate other reviewer’s comments.
8. There needs to be a citation in the sample size section for the cited evidence. Is the confidence interval a 95% interval? Those limits seem unlikely but I cannot check them without the citation.

As this is an explorative study sample size power calculation was not carried out hence the 95% intervals will be difficult to add.

9. ANCOVA is appropriate for continuous outcomes in RCTs. Are there any dichotomous outcomes?

We agree that ANCOVA is appropriate for RCTS but we have purposely avoided the "lethal sin" of analyzing changes from baseline for the statistically correct approach of ANCOVA adjusted for baseline measures. There are no dichotomous outcomes in the analysis.

10. Provide more details for the multiple imputation approach. How many draws? Are you drawing within participant? Or within follow-up visit? Any other considerations?

We have had further discussions with the statisticians and with such a small study multiple imputations will be of limited value. Indeed we considered using last observation carried forward but this is suggested to be best avoided while modelling is complex. Therefore we will analyse all available data without data replacement as these are missing at random rather than related to the trial intervention.

11. Why are oxidative stress, inflammatory markers and markers of AKI singled out for simple statistics and figures? Why not use ANCOVA?

ANCOVA which we recognise has limitations in this small sample size will be used and we will discuss this with the statistician.

12. There is a lot of text spent on one compliance issue in lines 334-340. This should be moved to the end of this paragraph and shortened considerably.

We have moved and shortened as suggested.
13. How will NGAL be analyzed in participants who have baseline markers for heightened inflammation or infection?

These patients have been excluded as IV iron is not administered during an active infection or in those patients on antibiotics or those with suspected infection. This is detailed in the exclusion under confounding factors in Table 1.

14. In Table 1, the inverse of the inclusion criteria need not be listed under exclusion criteria.

We disagree as this is normal practice and required by the ethics to ensure patients are appropriately included or excluded.

15. Table 2 needs footnoted a listing of expanded abbreviations. Will serious adverse events be assessed over the whole follow-up? Can this be added to Table 2.

We have added a foot note and yes SAE will be assessed over the whole period. This has been added to the table.

16. In Table 3, leave (SEM) in the title or as a footnote, and add units for Age and Serum Ferritin. Drop Mean before Age as you are using means throughout the table. Brackets are missing from the uPCR row. The number of decimal places is not always consistent.

Many thanks for pointing out the errors which have been corrected in the revised manuscript.

17. In Figure 2, 9 are not eligible. Should these be considered screen failures? Or did they not consent? The terminology makes it unclear.

We have revised the manuscript to clarify that these 9 patients were screen failures.
Reviewer #4: In this protocol, Ziedan et al propose a protocol and provide the baseline data for the prospective, open label assessment of the effects of three intravenous iron preparations on the markers of oxidative stress and kidney injury in kidney disease. This protocol rests on the hypothesis that the different iron preparations will result in variable generation rates of catalytically active iron, which in turn will affect oxidative stress, endothelial function and possible levels of kidney damage. The latter will be assessed by two markers of renal damage: proteinuria and levels of NGAL. The interventions include the use of three different preparations of iron (dextran, sucrose and isomaltoside) which will be delivered as infusions of 200mg for each product except the isomaltoside preparation in which 1000mg will also be used. Forty patients will be enrolled in this study and will be randomized 1:1:1:1 to the different iron preparations/doses. The (numerous) primary outcomes include measurement of markers of renal function, damage, endothelial function, inflammation and oxidative stress.

The premise of the study is an interesting one, considering the aggressive "marketing" of these pleotropic effects of iron preparations, the variable costs associated with each product and the renewed interest in iron therapy as an Erythropoietin Stimulating Agent sparing therapy.

The introduction is well written and provide an interesting summary of existing studies. The statistical methodology is well written and the authors avoid the "lethal sin" of analyzing changes from baseline for the statistically correct approach of ANCOVA adjusted for baseline measures. I have a few comments for the author consideration prior to publication of this study protocol

1) The small sample size justifies the labeling of this study as "explorative" and thus it is highly unlikely that the study will provide definitive data: at best it will demonstrate the feasibility of a larger randomized controlled trial in this domain. Although the authors acknowledge the exploratory nature of their study, they suggest that the primary objective of the study is to provide valuable information on the multiple outcomes (lines 154-158). This is highly speculative and in fact impossible. In my mind, the only thing that this pilot can show is that these outcomes can be collected; the small sample size precludes the generation of valuable information or inference. Please rewrite this section and also the statement in lines 371-372 of the discussion that alludes to the possibility of generating data on relative efficacy of these agents

We entirely agree with this comment and have revised the text to revise the more speculative nature of the results which might demonstrate reliability as measures and perhaps indicate a trend which might allow for a power calculation in a large study but not definitively.
2) I am somewhat concerned about the presentation of the study flow: whereas 521 were identified as potential participants, only 40% were contacted and eventually only forty were randomized. By design, the authors would not have been able to recruit and retain non-local participants (237 per line 332). This is a major limitation due to the complexity of the protocol that limits its "translation" to a bigger study. The authors may want to comment about this aspect in the discussion and offer ways in which the complexity of the study procedures may be reduced in a future replication.

Many thanks for this. We agree this highlights the importance of the feasibility of a future larger study for patients with the intense regime which may require revision to allow better recruitment. 40% of the 521 patients were identified as potential participants based on their stage of renal dysfunction (CKD stages 3-5) and their most recent Hb, serum ferritin and transferrin saturation. Many of these patients were receiving therapeutic iv iron at regular basis hence they were unlikely to meet the inclusion criteria, especially when they had iv iron within 6 weeks on the day they were identified as potential patients. The majority of patients recruited lived locally and were reviewed regularly at Hull Royal Infirmary. The rest were from nearby towns located within the East Yorkshire region. All patients were under the care of our regional nephrologist. Towns near Hull included, Driffield, Bridlington, and Hornsea.

3) The discussion should include a section on the future use of the data from this study and how it can be used to power/design a study which allow the investigators to compare these iron preparations in a statistically and clinically meaningful fashion

This has been added to the revised manuscript but is speculative of the results obtained. Again depending on the final outcome the design may need revision to allow feasibility. We believe that the future use of reliable data from this study will allow us design a larger study to permit investigators to compare these iron preparations in a more statistically and clinically meaningful manner.

4) The decision to use NGAL rather than the many other markers of renal toxicity investigated by the HESI and PSTC consortia and qualified by the FDA and EMEA as biomarkers of renal toxicity should be discussed. In fact NGAL is not one of the qualified biomarkers of kidney toxicity (see the papers by Fuchs et al https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3231866/ and Dieterle et al https://www.nature.com/articles/nbt.1622 which describe the qualified biomarkers (e.g. clusterin, beta 2 microglobulin, clusterin, Cystatin C, TFF3 and RPA-1). NGAL
is not a qualified biomarker at the present time. This is a major limitation of the study protocol concept since any regulatory implications of this study will only have to rely on one biomarker ie urinary albumin.

We thank the authors for pointing out this. One of the limitations was the cost in this study and the availability of the assays. Ideally if we had limitless funding and the expertise this may have been feasible. We do have data on cystatin C. we did not design the study as part of a regulatory approval but to understand better the possible differences in iron biology.

We hope that our combined use of functional and damage markers, will help advance the field of biomarkers of drug kidney toxicity in the future.

We hope the above-listed alterations render this document acceptable for publication.

Kind regards
In anticipation

Prof. Sunil Bhandari
Consultant nephrologists/Physician
Honorary Clinical Professor

Tel 01482674566
Fax: 01482674998
Email: Sunil.Bhandari@hey.nhs.uk