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Professors Josep M Campistol and Anil Mandal

Editors-in-Chief,

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Dear Sirs,

We would like to thank you for provisionally accepting our manuscript entitled "Diabetes mellitus increases the prevalence of anemia in patients with chronic kidney disease: a nested case-control study" for publication in the World Journal of Nephrology. The revised version of our manuscript has been updated according to the Guidelines and Requirements for Manuscript Revision-Case Control Study. You will find our numbered responses addressing the Reviewers' comments in the following pages. We have also performed a small change in the order of the study authors, for which all the authors agree, in order to be fair to the amount of effort put on this study. We hope you will accept this resubmission, as all the comments and suggestions, (coming only from Reviewer #4) s have been taken into account and we proceeded to revisions accordingly.

All the additions and changes made have been clearly indicated in the revised version of our manuscript by red colour. A clean copy of the revised manuscript is also submitted.

We are willing to provide any additional information you will probably consider necessary.

Yours Sincerely

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We would like to thank the Editors and the expert Reviewers for their comments and for their effort in reviewing our manuscript.

Each of their comments are addressed as follows

REVIEWER COMMENTS
Reviewer # 1 (code: 00503339)
Your careful study of CKD patients by stage does support the inferences and conclusions you proffered. The last step tin clinching your thesis would be a prospective study over time starting with CKD-2 to note the proportion with and without diabetes who become anemic. How diabetes results in worsened anemia has yet to be clarified. We would like to thank the Reviewer #1 for his effort in reviewing our manuscript. We agree with his comment that a next step can be another study with prospective follow of patients of CKD Stage 2 to identify the time sequence of anemia incidence in relation to the actual renal function.
Reviewer #2 (code: 00225280)
Key aspects: This study aimed to compare anemia prevalence between matched chronic kidney disease patients with and without diabetes mellitus and to assess factors associated with anemia development. This study adds to our knowledge the information that prevalence of anemia

is higher in diabetic than matched non-diabetic CKD patients and diabetes is independently associated with anemia occurrence. The new visions that the manuscript offers to readers is the a direct comparison in patients with and without DM in CKD, which was absent with the careful matching of individuals to form the two study groups This study further aimed to evaluate the possible association of demographic, clinical and laboratory factors with the development of anemia. The prevalence in patients with DM was about 15% higher than that in non-diabetic counterparts (47.8% vs 33.2%). Anemia is an established complication of CKD and is per se associated with the severity of renal insufficiency, The study further supports the principle indicated, progressing increase in prevalence of anemia with the progression of CKD from Stage 2 to Stage 4.The principal message is that detection and treatment of anemia in diabetic CKD patients should be performed earlier than in non-diabetic counterparts. The overall structure of the manuscript is complete and truly facilitate the progress of knowledge in the relevant field. The manuscript provides adequate details of methods, the source of the data that is presented are reliable. Weakness: Limitations of this study include the cross-sectional observational study nature of the study. The use of a unique hemoglobin measurement to determine the diagnosis of anemia Overall levels of enthusiasm: high, because there has not been published research with a careful matching of individuals to form the two study groups

We would like to thank the Reviewer #2 for his comments and for his effort in reviewing our manuscript.

Reviewer #3 (code: 00503272)			
Good work! We would like to thank the Reviewer #3 for his comment and for his effort in reviewing our study.			
Reviewer # 4 (code:00502999)			
This paper by Skodra et al. is about the association between diabetes mellitus and anemia in chronic kidney disease. One of the main strengths of the paper is that is follows a prospective, well-organized nested-case control design.			
No	**Comment**	**Response**	**Page in the Revised Manuscript**
1)	Reviewer #4 commented: "However, the word Prospective is not addressed in the METHODS section of the Abstract. Please include."	We thank the Reviewer #4 for this suggestion. We have now changed the relevant phrase to "This is a nested case-control study of 184 type-2 diabetic and 184 non-diabetic CKD patients from a prospectively-assembled database..."	Abstract: Page 3

2)	Reviewer #4 commented: "Results: Delete insignificantly and replace it by non-significantly."	We appreciate this comment from the Reviewer #4. We have now changed the word "insignificantly" to "non-significantly" in the Abstract section of our revised manuscript.	Abstract: Page 3
3)	Reviewer #4 commented: "Results: The 4th and 5th line contains a sentence which is not well written. Is the word "iron" missing after "...while serum...was similar..."?"	We would like to apologize for this typographical error. The word "iron" has now been added in the relevant phrase.	Abstract: Page 3
4)	Reviewer #4 commented: "Conclusion: please address that the prevalence of anemia varies with the stage of CKD."	We thank Reviewer #4 for this comment. We have changed the phrase "Prevalence of anemia is higher in diabetic than..." from the original paper to "Prevalence of anemia progressively increases with advancing Stages of CKD and is higher in diabetic than..." in the revised version of our manuscript.	Abstract: Page 3
5)	Reviewer #4 commented: "Introduction: In the second paragraph of page 5 please	We appreciate this comment from the Reviewer #4. Following his suggestion, we now change the phrase "Diabetes Mellitus (DM) is proposed to elevate the risk…"	Introduction: Page 5

	state that diabetes is the main cause of ESRD worldwide."	from the original manuscript to "Diabetes Mellitus (DM) is the leading cause of CKD and ESRD and is proposed to elevate the risk…". An appropriate reference (Saran R, Am J Kidney Dis 2016) has also been added.	
6)	Reviewer #4 commented: "A drawback of the study is that diabetes Type 1 cases have not been differentiated from type 2 cases. In this regard, is the relationship between Type 1 diabetes and anemia the same as with Type 2 subjects?. This important question must be discussed. If the authors have this data available, it ought to be included If not, discuss this point and address it as a limitation of the study."	We thank the Reviewer for this comment. In this study, type 1 diabetics were excluded from the analysis, for exactly the reason the Reviewer implies, i.e. patients with type 1 DM were too few. Therefore, they could not be analyzed separately and since we wanted to have a uniform population we decided to exclude them in advance. We kindly refer the Reviewer to Materials and methods section of our original manuscript (please see Study design, Page 6) in which we specifically mentioned that "*Exclusion criteria were type 1 DM, Stage 5 CKD (eGFR <15 mL/min/1.73m^2) or kidney transplant.*"	Page 6

7)	Reviewer #4 commented: "Did the level of glycemia correlate with the degree of anemia?"	We appreciate this comment from the Reviewer #4. In order to answer this question, we have now performed again the uni- and multivariate logistic regression analyses presented in Table 3 so that serum glucose levels are also included. As shown in revised Table 3, in the univariate analysis increased serum glucose levels were significantly associated with anemia occurrence (OR: 1.006, 95% CI: 1.002-1.011), but this effect disappeared in the multivariate analysis (OR: 0.999, 95% CI: 0.992-1.005), where the presence of DM per se was also taken into account. Therefore, the level of glycemia was not independently related with anemia prevalence, whereas presence of DM was. Furthermore, inclusion of glucose levels in the multivariate analysis did not practically change our original results, with the exception of some minor changes in the effect measures (ORs) and CIs, now depicted in red in the Table and the Text of our manuscript.	Page 26
8)	Reviewer #4 commented: "What is the role AGEs and other glycosylated	We thank the Reviewer #4 for this comment. There are a few earlier studies evaluating the role of AGEs in anemia development; they suggest that elevated	Page 12

	molecules play in the development of anemia?"	levels of serum AGEs are associated with anemia occurrence in general; some underlying pathophysiologic mechanisms have been proposed, but are not definite. In an observational study including 519 women aged 65 and older, patients with anemia (defined as hemoglobin<12 g/dL) had significantly higher levels of serum carboxymethyl-lysine (CML) [anemic: 0.56 (0.46 to 0.76), non-anemic: 0.54 (0.44 to 0.65); p=0.0018] [1]. Moreover, in this study, patients with anemia of chronic kidney disease (eGFR <30 mL/min/1.73 m^2) had the highest levels of CML compared to patients without anemia and patients with anemia caused by nutrient deficiency, chronic inflammation or unexplained anemia [CKD, 0.85 (0.60 to 1.89), Non-anemic 0.54 (0.44 to 0.65), Nutrient deficiency 0.54 (0.44 to 0.71), Chronic inflammation 0.58 (0.47 to 0.73), Unexplained anemia 0.60 (0.46 to 0.78) µg/mL; p=0.002] [1]. In another study in patients with type 2 DM, low-molecular-weight (LMW) AGEs were significantly associated with glomerular filtration rate	

		(GFR) and hemoglobin (both P<0.001), while eGFR was the strongest determinant of LMW AGEs levels (P<0.0001) and patients with renal impairment and anemia had the highest LMW AGEs levels [2]. Anemia occurrence due to AGEs accumulation is rather not mediated by iron metabolism impairment or systemic inflammation (measured by CRP), as neither of these was associated with the levels of AGEs in previous studies [2]. The increased levels of AGEs observed in erythrocytes compared to other cell lines have been associated with increased erythrocytes deformability and thus decreased cellular life due to mechanical damage [3]. Moreover, in patients with DM, accumulated AGEs in erythrocytes' extracellular matrix bind with specific AGEs receptors on the vascular endothelium inducing oxidative stress which increases erythrocytes damage and results in cellular apoptosis [4]. However, it is also known that reduced tissue oxygenation is associated with anemia and contribute to the formation of AGEs [5]. Therefore, it is possible that a	

		bidirectional cause-and-effect relationship exists between AGEs and anemia occurrence. In order to comply with this comment and to indicate the role of AGEs in anemia in diabetic patients with CKD, we now add a phrase (keeping in mind the word limits of the journal) in the relevant 4th paragraph of the Discussion section of our revised manuscript, so that one would read: "*advanced glycation end products (AGEs) possibly decreasing erythrocyte lifespan.*" The relevant references have also been added. <u>References</u> 1. Semba RD, Ferrucci L, Sun K, Patel KV, Guralnik JM, Fried LP. Elevated serum advanced glycation end products and their circulating receptors are associated with anaemia in older community-dwelling women. Age Ageing 2009; 38: 283-289 [PMID: Pmc2724885 DOI: 10.1093/ageing/afp011]	

2. Thomas MC, Tsalamandris C, MacIsaac R, Medley T, Kingwell B, Cooper ME, Jerums G. Low-molecular-weight AGEs are associated with GFR and anemia in patients with type 2 diabetes. Kidney Int 2004; 66: 1167-1172 [PMID: 15327413 DOI: 10.1111/j.1523-1755.2004.00868.x]

3. Iwata H, Ukeda H, Maruyama T, Fujino T, Sawamura M. Effect of carbonyl compounds on red blood cells deformability. Biochem Biophys Res Commun 2004; 321: 700-706 [PMID: 15358163 DOI: 10.1016/j.bbrc.2004.07.026]

4. Wautier JL, Wautier MP, Schmidt AM, Anderson GM, Hori O, Zoukourian C, Capron L, Chappey O, Yan SD, Brett J, et al. Advanced glycation end products (AGEs) on the surface of diabetic erythrocytes bind to the vessel wall via a specific receptor inducing oxidant stress in the vasculature: a link between surface-associated AGEs and diabetic complications. Proc Natl Acad Sci U S A 1994; 91: 7742-7746 [PMID: 8052654]

5. Thomas MC, MacIsaac RJ, Tsalamandris C, Power D, Jerums G. Unrecognized

		anemia in patients with diabetes: a cross-sectional survey. Diabetes Care 2003; 26: 1164-1169 [PMID: 12663591 DOI: 10.2337/diacare.26.4.1164]	
9)	Reviewer #4 commented: "The statistical methodology is appropriate. Do the authors think HbA1c levels could have added more information to the presented conclusions?"	We appreciate this comment from the Reviewer #4, this is relevant to his/her comment #7 above. It is possible that HbA1c levels could add some more information, as in the case of glucose levels. We have to note, however, that this is a nested case-control study, deriving from a population of "*CKD patients first visiting a Nephrology Outpatient clinic*", and we wanted to test prevalence of anemia in these two groups in comparison. As this was not a Diabetes Clinic, HbA1c levels have not been routinely measured and we cannot answer this comment.	
Reviewer #5 (Reviewer's code: 00503203)			
The study deals with a common issue in clinical practice; (i.e. diabetic patients with moderate CKD often appear with low Hb levels for their eGFR levels and have already been investigated for anemia from internists or hematologists for years with no results). Although there are some previous data pointing to the fact that anemia (among many factors studied) is more common in diabetics with CKD, this study adds to current			

knowledge.

We would like to thank the Reviewer #5 for his effort in reviewing our manuscript.