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Answers to Reviewer comments

We sincerely appreciate all the comments and suggestions by both esteemed reviewers. Through their insightful recommendations we have amended the manuscript and filled the gaps in our hypothesis. We now explain how the ACE2 may interact in children irrespective of genetic background. We discuss EPO in high altitude and Bartter's syndrome that offer natural human models to test our hypothesis. We have added a discussion why EPO is blunted or unresponsive in adults in general and especially in ACE D allele carriers.

Below please find point by point answers to the Reviewers' comments:

Answers to Reviewer 1:

Main point: The status of overall research: Our hypothesis offers an encompassing, Occam's razor, explanation that pieces together most if not all aspects of the current COVID-19 puzzle. RAS system overactivity has been proposed, ACE I/D polymorphism associations have been evaluated, Ang II and EPO levels have been measured and reported, IL-6 and PAI-1 have been heavily discussed and treatments developed and applied. We do refer to and discuss all those aspects in all the different sections and bring all together into one common pathway, EPO secretion and role as "a phylogenetically preserved ancestral neuroprotective innate immune response mechanism preventing lethal cerebral damage from both non-communicable (kernicterus, prematurity) and communicable insults (cerebral malaria)". We bring in the Thalassemias (that augment EPO) and FGF23 (that depresses EPO and vitamin D, the latter so much discussed in relation to COVID-19…), as two aspects that solidify the central role EPO plays. To date, FGF23 has not been evaluated in COVID-19 which strengthens our position that our integrative hypothesis includes innovative, not previously considered aspects.

1. We accept (and have been about to propose it as well) a **change of title** to: "**Age and Genotype Dependent Erythropoietin (EPO) Protection in COVID-19.**" We believe this new title better reflects the main points of this minireview.
2. Changed to a similar version as suggested; now reads: "The severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), the cause of the coronavirus disease of 2019 pandemic (COVID-19) has to date (June 23, 2021) infected almost 180 million people worldwide, causing nearly 4 million deaths[1]. The COVID-19 pandemic continues to be a global threat despite increasing vaccinations[1]."
3. Corrected format as per World Journal of Stem Cells.
4. Reference one corrected and all references are changed according to the WJSC format.
5. Additional points *(reviewer comments in italics)* **Answers:**
    a. *antiapoptotic and cytoprotective properties of EPO:* We have added a two-sentence discussion on EPO treatment and acute lung injury (ALI)/acute respiratory distress (ARDS) [Kakavas et al: ref 20] and "Cytoprotective effects of erythropoietin: What about the lung?" by Haine et al [ref 21].
    b. *potentiate mobilization of iron stores:* We have chosen not to discuss mobilization of iron stores as there is an intricate connection between iron metabolism, EPO and FGF23 that would confuse the reader at this point. It could be the topic of another minireview once our hypothesis gains more traction. Whatever disturbances in iron levels are seen in COVID-19 are due to an acute inflammation (low iron but no real iron deficiency and increased ferritin) and would be restored once EPO subdues the inflammation, potentially restoring overall homeostasis. Replenishing iron without considering FGF23 are known to lead to hypophosphatemia and further FGF23 increases [Ref 138] which could theoretically impact COVID-19 outcomes negatively.
    c. *causing interferon production:* Ref 26 reports INF-gamma decrease and [Araki D et al. *Blood Cells Mol Dis* 2020;85:102488 PMID: 32889151 DOI:

10.1016/j.bcmd.2020.102488] reports that INF-gamma induces an inflammatory block to EPO responsiveness which would further strengthen our hypothesis. We have chosen not to discuss it in this manuscript.

d. *stabilizing endothelium, protecting the integrity of the pulmonary epithelial cell and attenuating pulmonary edema:* see a. above and Kakavas et al [ref 20] and Haine et al [ref 21].

e. *Additionally, are there any results of cases report or clinical trials of EPO in COVID-19 so far:* We have already referred to one case report (ref 123) and added reference 41 reporting on low EPO at 4,500m in patients with fatal COVID-10. Also added, additional references [ref 124-126] to publications suggesting EPO treatments.

Answers to Abhinav Sinha **Reviewer 2:**

General comment *(reviewer comments in italics)*: *However, there were many instances in the entire MS where the authors tried to over-simplify the issues, particularly those involving ACE and ACE2 polymorphisms and their effect on Angiotensin II levels.*
Answer: We have amended the abstract and core tip and added extensive discussion on ACE2 in pages 8 to 11 and 14, as well as added necessary and suggested references that reflect how ACE2 contributes to the etiopathogenesis. We will detail the additions at specific points in the discussion below.

Abstract

1. *genetic* changed to: selective.
2. *Emerged* changed to: positively selected.
3. It was suggested that the sentence "*When malarial and other cerebral threats abate and the young child survives to adult age, EPO subsides*" was unclear.

Answer: Here we mean that it is congruent to the neuroprotective function of EPO that once the child grows up it is understood that the threats have subsided, and EPO should thus decline. In O'Donnell et al. [ref 25], the EPO increase seen in severe malaria is not observed anymore in older children or in adults with beta thalassemia irrespective of malarial infection.

4. *Predisposed with what?* **Answer:** It is meant those carrying the D allele. This sentence has been changed to a few clearer sentences as seen to make it clearer in the new paragraph of the abstract. The discussion now contains references to the ACE2 input in the etiopathology as well.

Core tip

5. Amended as it appears now in the new version. The language has been simplified, and we believe its flow is better.

Introduction

6. Changed as suggested to: associated with mild malaria versus severe malaria
7. Added as suggested: vs. the I allele
8. Changed *claim* to: posit

ERYTHROPOIETIN'S TISSUE PROTECTIVE ACTIONS

9. Added discussion and references about EPO distribution (ref 10)
10. Changed *how* to: the way

YOUNG AGE AND EPO AUGMENTING GENETIC DETERMINANTS

11. *Unless EPO is pharmacologically and exogenously…,* **Answer:** changed to: "Endogenous EPO, dissonantly elevated from what is expected by a concurrent anemic stimulus and presumably to exert its non-erythropoietic tissue protective functions, has been reported in few studies."
12. *ACE I/D polymorphism* changed to "the ACE D allele"

13. *The statement may not be "generalized" as the study mentioned in ref 5 was done in malaria patients whereas the statement reflects the ACE D allele distribution in "areas of high malaria burden" which implies general population living in high malaria burden areas…*; **Answer:** changed to "The ACE D allele, also significantly associated with milder forms of malaria in areas of high malarial burden, is another sophisticated genetic selection" and added 1 reference [ref 5, 27-29]
14. *Complicated sentence…please break down*; **Answer:** changed to two sentences: The wide distribution of RAS in every human organ and the presence of the ACE D allele ensure that adequate substrate, and enzyme levels (ACE) are abundant[30,31], to provide for locally elevated Ang II levels sufficient for its paracrine effects on EPO secretion and stimulation[12,32]. In addition, Ang II may exert immune system modulation[33] and/or direct anti plasmodium activity[34].
15. *The references cited here for EPO protecting against invasion do not justify the claim. Authors are requested to provide more specific references for this*; **Answer:** Ref 26 reports that EPO protects against murine cerebral malaria through actions on host cellular immunity. Ang II also exerts immunological effects in malaria [ref 33] and Ang II (and derived peptides) demonstrated anti-plasmodial activity [ref 34]. Ang II induced EPO expression **in situ** in murine kidney slices and in 786-O kidney cells **in culture** and current data suggest that Ang II, in addition to regulating blood volume and pressure, may be a master regulator of (non—hypoxic) EPO secretion and erythropoiesis [ref 32]. It is therefore, tempting to speculate that (systemic and/or local tissue) Ang II-EPO interactions are involved. There is overwhelming evidence for local tissue RAS in virtually organ/tissue [ref 30,31]. Local tissue EPO mRNA has been detected in similarly numerous sites and is under hypoxia and possibly RAS regulation [10,11,12,14,21,32]. In ref 12 there is evidence of a local RAS in bone and bone marrow participating in hematopoiesis. Moreover, exogenous

EPO alleviates post-resuscitation myocardial dysfunction in rats potentially through increasing the expression of angiotensin II receptor type 2 in myocardial tissues through local myocardial RAS [ref 35]. In [Malikova E et al. Mol Cell Biochem. 2016; 418(1-2):147-57. PMID: 27344167 DOI: 10.1007/s11010-016-2740-z], the coexistence of EPO mRNA and Renin is reported in the lung, "*and the greatest elevation of erythropoietin was present in tissues with the highest renin levels. It is known that angiotensin II can stimulate EPO expression; therefore, RAS activation in the monocrotaline group likely acts in synergy with renal hypoperfusion and hypoxia to stimulate EPO expression. Furthermore, the stimuli that promote renin and EPO expression partly overlap.*" Thus, the existence of local tissue RAS and tissue EPO systems is real and functional. Local EPO increase can be beneficially regulated by both local/paracrine and systemic/endocrine Ang II. Systemic Ang II could beneficially exert a local EPO increase, reportedly advantageous for the brain as systemic EPO difficultly crosses the blood-brain barrier and a local EPO increase would be swifter [ref 21]. We have however changed the earlier sentence to: "to provide for systemically and/or locally elevated Ang II levels sufficient for endocrine or paracrine effects on EPO secretion stimulation[12,32]. In addition, Ang II may exert immune system modulation[33] and/or direct anti plasmodium activity[34]. The subsequently increased local tissue EPO levels would thus bypass systemic EPO prothrombotic effects while possibly also conferring the demanded tissue protection[35] and Plasmodium invasion mitigation[12,26,32]." and changed the references to correspond to the above discussion.

<u>EPO IS AN ANCESTRAL NEUROPROTECTIVE MECHANISM PREVENTING LETHAL CEREBRAL INSULTS AT YOUNG AGE: implications for COVID-19</u>

16. *Younger years* changed to: younger age groups
17. *Conditions* changed to: insults
18. *Oversimplification on ACE/ACE2 balance. More discussion on ACE2 effects*.
    Answer: We added the paragraph: "It is conceivable that evolution uses the ACE2 as a "bait" for SARS-CoV-2 to gain entry in order to trigger an ACE/ACE2 imbalance[42-44] and stimulate EPO hypersecretion using RAS, uncoupled from hemoglobin levels. Low nasal ACE2 levels present in children[37] would beneficially intensify this imbalance, especially for those without protective genetic determinants[37]. Genetically predisposed children already enjoy protective EPO levels through sustained elevated Ang II levels, through the ACE D allele in some, the ACE2 T allele leading to lower ACE2 expression in females[5,45], or HbE/beta thalassemia in others, thus protecting against coronavirus disease 2019 (COVID-19), in similar ways seen in malaria and dengue fever[46] (Figure 1)." and added more discussion on ACE2 effects as suggested.
19. *a-:* changed to α-thalassemia
20. Rephrased as suggested to: the frequency of ACE D allele was significantly higher (57.9%) in mild malaria patients as compared to that in severe malaria patients[5].
21. The sentence: "*However, EPO levels seem to decline naturally in a similarly protective manner at older ages.*" **Answer:** Removed and replaced by: "Longitudinal studies show an overall EPO level decrease with increasing age, but the influence of the ACE D allele/DD genotype on EPO decline is not known;"
22. *If the raised EPO has genetic determinants, as claimed, how would raised EPO be normalized in adults belonging to the same geography?* **Answer:** Longitudinal studies on EPO shown declining levels in the general population but no studies report on EPO with Mendelian randomization. However, a study in adults with non-small cell lung cancer shows higher EPO levels in patients

with the D allele[ref 56]. Raised EPO levels correlate with increased morbidity and mortality in various conditions that also show significant associations with the D allele. It is possible but has not been studied whether EPO is a marker for the presence of the ACE D allele, and the risks are mediated through Ang II effects. We have replaced the above sentence with: "Most, if not all the above conditions share associations with the ACE D allele[54] and thus, elevated EPO levels in non-anemic individuals maybe a marker for the presence of the D allele and the elevated Ang II it subsequently encodes[7,8,55,56]." that clarifies the intent of our statement. Finally, in ref 53 it is discussed whether EPO increase is also a protective response to proinflammatory cytokines and not per se the mediator of detrimental effects. In summary, the significance EPO has as a risk determinant is being currently investigated.

23. *In the sentence:* "It is thus at older ages that the EPO augmenting genetic determinants sustaining chronically elevated endogenous EPO levels may become detrimental for survival due to EPO's association with increased morbidities[5,21,22,36*]." Refs 5,21,22 didn't mention about EPO.* **Answer:** We have replaced this sentence with the sentence: "Most, if not all the above conditions share associations with the ACE D allele[54] and thus, elevated EPO levels in non-anemic individuals maybe a marker for the presence of the D allele and the elevated Ang II it subsequently encodes[7,8,55,56]."
24. *Should also mention the roles of ACE2 in "The malarial protection engendered by the EPO augmenting ACE I/D polymorphism…."*. **Answer:** changed to: "The malarial protection engendered by the EPO augmenting ACE I/D polymorphism and the ACE2 T allele[5,45]," with appropriate references added.
25. *Increased mortality.* **Answer:** Changed to the following sentence: "The association of HT with higher risk of severe or fatal COVID-19[60] and association of HT with the ACE D/ACE2 T alleles reported in several Indian

populations[45,61,62] could explain the statistics observed in India during the current phase of the COVID-19 pandemic[1]."

THE ACE D ALLELE / DD GENOTYPE AND EPO INTERPLAY: implications for COVID-19 (Figure 2)

26. *the over-expression of the ACE receptor*. **Answer**: removed. Wrong wording.
27. *In the presence of the ACE D allele/DD-genotype, the over-expression of the ACE receptor in combination with ACE2 downregulation due to SARS-CoV-2 infection, would result in ACE/ACE2 imbalance.* **Answer***:* We have added ACE2 in the discussion and changed to: "Congruent to its primary evolutionary (neuroprotective) objective of EPO secretion enhancement when threatened by pathogen invasion, ACE D allele/DD-genotype elevated levels of Ang II, reduce ACE2 tissue expression and activity by stimulation of lysosomal degradation through an Ang II type 1 receptor (AT1R) dependent mechanism and thus, might mitigate entry of pathogens using the ACE2 receptor[77,78]. The ACE2 malaria protective T allele could further reduce ACE2 expression and similarly mitigate pathogen egress[45]. ACE2 is ubiquitous and also present in type I and type II alveolar epithelial cells[77,79] and loss of ACE2 expression as with increasing age, in males and type 2 diabetes (DM)[81],is known to precipitate severe acute lung failure[81]. Binding and internalization of ACE2 by SARS-CoV-1/2 is using the same AT1R dependent mechanism as Ang II[44], in reducing ACE2 cell surface expression[42,43]. A vicious circle of ACE/Ang II/ACE2 imbalance and persistently increased Ang II levels through continual RAS over-activation might lead to lung shut-down, in similar mechanistic ways also described in human H7N9[82] and H5N1[83]. Additionally, an aberrant T- cell-mediated immune response and cytokine storm could further be mediated by the excessively elevated and unopposed Ang II levels [63,83]."

28. *In both ACE I/D and ACE2 snp, the I allele is associated with reduced ACE (as compared to D allele) and the C allele are associated with increased ACE2 (as compared to the T allele). Hence, assuming no selection due to malaria, the genotype would be ACE I allele (which decreases ACE = low Ang II) and ACE2 C allele (which increases ACE2 = low Ang II), would the person be hypotensive (assuming that ACE D and ACE2 T would predispose to HTN)?? What will be the ACE/ACE2 allelic composition in the normotensive persons then??* **Answer:** Patel SK et al. state that plasma ACE2 activity levels are low in healthy individuals and may or may not be increased in patients with hypertension. Males have higher circulating ACE2 activity than females, but few reports have analyzed data according to gender[Patel SK et al. From gene to protein-experimental and clinical studies of ACE2 in blood pressure control and arterial hypertension. Front Physiol. 2014; 5: 227 PMID: 25009501DOI: 10.3389/fphys.2014.00227]. The most recent study reported similar mean plasma ACE2 concentrations at 5.41 in men and 5.13 in women[Nelson CP et al. Genetic Associations With Plasma Angiotensin Converting Enzyme 2 Concentration: Potential Relevance to COVID-19 Risk. Circulation. 2020; 142(11): 1117-1119 PMID: 32795093 DOI: 10.1161/CIRCULATIONAHA.120.049007]. The same study identified 3 loci that explained 4.91% of variation in plasma ACE2 concentration in men and 1.14% in women hypothesizing sex-specific genotype effect on plasma ACE2 concentration[Nelson et al as per above]. It seems thus that ACE2 polymorphisms despite a gender-genotype effect cannot explain plasma ACE2 variations as the ACE I/D polymorphism that explains over 28-44% of plasma ACE variability[ref 55, Rigat et al. 1990]. Normotension is thus not dependent on p-ACE2 level association to any genotype.
29. *"In COVID-19 infection and under the influence of the ACE 'D' allele and the excessively increased Ang II levels[85], caspase-1 mediated pyroptotic inflammatory cell necrosis…". See the comment above in 28. Has anyone demonstrated ACE and ACE2 levels in ACE I/D and ACE2 C/T polymorphisms together with Ang II levels?*

Whats the normal physiological levels of these two enzymes and what is the genotypes under physiological conditions?? **Answer:** As per 28 (Patel SK et al 2014; Nelson CP et al 2020). ACE I/D polymorphism explains up to 44% of the plasma-ACE while only 1.14-4.91% of plasma-ACE2 variability is explained by ACE2 polymorphisms. Thus Ang II levels are overwhelmingly under plasma-ACE control as reported by references 7 and 8 in the final manuscript.

30. *ACE D allele induced Ang II elevation is not shown in the mentioned reference number 68.* **Answer:** Indeed, we showed plasma-ACE D allele dependent increase but did not measure Ang II. The sentence is changed to: "This pattern is analogous to our findings in sarcoidosis where ACE D allele induced serum ACE increase *and subsequent Ang II elevation* can steer the immune system…"
31. *"The chronically elevated Ang II in COVID-19SARS-CoV-2- infected adults with the ACE 'D' allele seem indeed…": Has it been studied/reported or hypothesized?* **Answer:** This sentence has been deleted and replaced with a discussion on endogenously elevated EPO levels and their relations to COVID-19 prevalence, EPO levels in high altitude COVID-19 as well as in human genetic models. The regulation of EPO on kidney level is also discussed bringing insights in why EPO is not elevated in the face of high Ang II levels {ref106].
32. *Better to write the name of the component (hormone/protein/enzyme).* **Answer: changed to** 1,25-dihydroxyvitamin D3.
33. *See above comments and please mention the threshold for elevation of FGF23 through Ang II to be able to say that it is ANg II induced and not by anything else.* **Answer:** it is clearly stated in ref 116 (de Cavanagh EM et al) that "Angiotensin II reduces renal Klotho expression, which interferes with FGF23 signaling and results in elevated FGF23 levels. In turn, the increased FGF23 content inhibits 1α-hydroxylase, leading to the lowering of 1,25-(OH)2D3 production."
34. *"…exogenous EPO influence FGF23 levels…". In what directions?* **Answer:** As stated in ref 118, 127: Human data show that both endogenous and

exogenous EPO influence FGF23 levels via alterations of the ratio of active to inactive FGF23 in favor of its inactive form, thus attenuating effects of bioactive intact FGF23 levels and could explain EPO's protective effects[118,127].

CONCLUSION AND THERAPEUTIC CONSIDERATIONS

35. *Long sentence. Need to reconstruct the sentence.* **Answer**: The long sentence has been reconstructed in to two sentences as per below: "The age dependent EPO secretion[22-25] and the contribution of the EPO augmenting genetic determinants in children and adults as a disease modifier in malaria is established[5,25-28]. In the present work, we posit that it extends to and explains COVID-19 protection in children[39] and can provide new pathophysiological insights and therapeutic avenues in adults."
36. "…excessively augmented in the presence of the ACE D allele,…" *Was that reported in any of these studies?? Or is it hypothesized?* **Answer**: It is a fact that Ang II is increased in COVID-19 [ref 84] and it is a fact that the D allele predisposes for severe COVID-19 [ref 59]. References 7 and 8 report the increased Ang II in the presence of the ACE D allele. References 67-70 all report on the ACE D allele related increases of IL-6 and PAI-1.
37. **In preparation for the proposal of EPO interventions, the following paragraph has been added to discuss existing treatment strategies and highlight inadequacies.** "Currently, therapeutic approaches are symptomatic and include empirical immunosuppressive and anti-inflammatory tactics (dexamethasone)[128], interferons[129], targeting of individual cytokines (IL-6: tocilizumab/statins/heparin; PAI-1: statins, and numerous target substances in development)[75,130-132] and corrections of isolated laboratory abnormalities (sodium disturbances)[133]. Prolonged use of these interventions may lead to serious adverse effects and reduction of host defenses with resurgence of opportunistic infections. An Occam's razor therapeutic strategy guided by mendelian, and mechanistic evidence should

instead be pursued. ACE I/D polymorphism genetic testing could be predictive and guide patient triage and treatment decision making as individuals with the DD genotype are predisposed for a more severe disease[59].

38. *How exogenous EPO would mitigate ACE 'D' allele mediated "increased" Ang II?* **Answer**: The evidence on EPO impacting on IL-6, PAI-1, FGF23, in multi-trauma patients and acute lung injury, ARDS is overwhelming regardless of genetic association to the ACE D allele. We have rephrased our intended meaning as: "Research evidence supports the notion that endogenously[109,112] and exogenously increased EPO levels[123] could break the vicious circle of persistent ACE D allele augmented Ang II stimulation on PAI-1, IL-6 and FGF23 by both synergistic and individual inhibition[20,21,122,123,127,134]."
39. *How similar or different is the rhEPO in comparison to the endogenous EPO? What's the difference between this (EPO derivatives) and rhEPO?* **Answer**: In references 10, 14, 134 the differences are described in detail. From ref 134 differences in rhEPO: epoetin-α, epoetin-β, epoetin-ϫ, epoetin-ω, epoetin-δ, and darbepoetin-α exhibit the same polypeptide chain of 165 amino acids, identical to endogenous EPO, but differs in carbohydrate structure. Darbepoetin-α is a hyperglycosylated molecule that stimulates erythropoiesis by the same mechanism as the endogenous EPO. ARA-290 and cEPO are non-erythropoietic EPOs binding to the TPR.
40. "*It is conceivable that this effect could also be replicated in COVID-19 vaccinations, especially in immunocompromised patients." How? Need more explanation on this.* **Answer**: Added explanation of the above statement: Moreover, in hematologic patients, rhEPO treatment was associated with an enhanced antibody response to the influenza vaccine, similar to that of healthy subjects and it is conceivable that this effect could also be replicated in COVID-19 vaccinations, especially in immunocompromised patients[135].