

## Response to Reviewers

We appreciate your careful review of our manuscript and hope that we have satisfactorily addressed each of the issues you have raised.

*Reviewed by 00503339*

Worthwhile study of a reasonable number of patients undergoing maintenance hemodialysis with attention focused on use of Regional saturation of oxygen (rSO<sub>2</sub>) as a potential measure of satisfactory attention to whole body status during Maintenance Hemodialysis.

The one "control" needed for grasping the full significance of a decreased oxygen (rSO<sub>2</sub>) in an ongoing dialysis patient is exploring whether decreased values might be related to anemia rather than insufficient duration of dialysis per se. Overall, proposal of a fresh variable to help explain unexpected severity of anemia in patients on maintenance hemodialysis is worth exploration, especially to note whether anemia due to an cause, might be sufficient to alter oxygen saturation (rSO<sub>2</sub>) in otherwise well dialyzed patients. Provocative and worthwhile.

**Response:**

We are very appreciative of your thoughtful comments and suggestions. The mean Kt/V and hemoglobin (Hb) concentration in this study were  $1.18 \pm 0.04$  and  $9.9 \pm 0.1$  g/dL, respectively. We found that the regional saturation of oxygen (rSO<sub>2</sub>) in lower-limb muscle was not associated with either Hb concentration or Kt/V. However, as the reviewer suggests, it is indeed important to clarify whether rSO<sub>2</sub> worsens with severe anemia, and whether blood transfusion can ameliorate this. We hope that our future work can address this question.

*Reviewed by 02505674*

The Authors performed a very interesting and well conducted study aimed at evaluating the factors influencing regional muscular oxygenation in patients underwent hemodialysis. The adopted statistical approach is convincing and the conclusions are supported by the findings reported. I have no remark on the study.

**Response:**

We really appreciate your careful review of our manuscript.

*Reviewed by 00289581*

Factors affecting oxygenation of lower-limb muscle tissue in hemodialysis patients: muscle oxygenation is associated with nutritional status, including serum inorganic phosphate and albumin concentrations Haruhisa Miyazawa, Susumu Ookawara, Kiyonori Ito, Katsunori Yanai, Hiroki Ishii, Taisuke Kitano, Mitsutoshi Shindo, Yuichiro Ueda, Yoshio Kaku, Keiji Hirai, Taro Hoshino, Kaoru Tabei, Yoshiyuki Morishita This is an observational study which looks at the relationship of lower-limb muscle oxygenation in hemodialysis patients and attempts to identify the factors affecting muscle oxygenation.

Main problems:

- 1) The investigators did not perform arterial vascular studies prior to the oxygenation score. Subclinical or clinical arterial disease could be responsible for these findings.

**Response 1:**

Thank you for your comment. As pointed out by the reviewer, we were unable to perform comprehensive arterial vascular studies for all the patients included in this study. However, we were able to ascertain whether the patients had intermittent claudication and cold lower-limb. Hemodialysis (HD) patients with these symptoms underwent angiography and/or magnetic resonance imaging to investigate the presence of peripheral artery disease (PAD). Therefore, we could and did exclude HD patients with symptomatic PAD in our study; however, arterial vascular studies were not routinely performed in HD patients without symptoms of PAD. Therefore, it is possible that HD patients with subclinical PAD were included in the study. We have noted the necessity of performing arterial vascular studies to exclude the influence of subclinical PAD, and will do so in future studies. We have revised the discussion section of our manuscript accordingly to include these details.

Page 10, Lines 14-26:

“HD patients with symptomatic lower-limb ischemia who were diagnosed with peripheral artery disease by angiography and/or magnetic resonance imaging were excluded from the study. The prevalence of subclinical peripheral artery

disease in HD patients is approximately 20-25% [4]. We did not use methods such as the ankle-brachial pressure index to investigate the presence of peripheral artery disease in asymptomatic patients. Therefore, while we have excluded patients with symptomatic peripheral artery disease from the present study, we may have inadvertently included some with subclinical disease. In these patients, muscle rSO<sub>2</sub> might be reduced via the decrease in oxygen supply caused by the microcirculatory impairment due to subclinical peripheral artery disease. Thus, it would be necessary to perform arterial vascular examinations in future studies so that such patients can be excluded."

Page 12, Lines 4-11:

"This study has several limitations. Firstly, its sample size was relatively small. Secondly, we could not evaluate the association between lower-limb muscle rSO<sub>2</sub> and calculated nutritional markers, including normalized protein catabolic rate and body mass index, anthropometric measurements representing nutritional status, and the severity of protein-energy wasting. Finally, we did not routinely examine the arterial vascular status of HD patients without symptoms of peripheral artery disease. As such, it is possible that some HD patients with subclinical peripheral artery disease may have been included in this study."

2) How did you determine the number of patients and controls to do?

**Response 2:**

We performed an initial statistical power analysis for the comparison of the lower-limb muscle rSO<sub>2</sub> between HD patients and healthy controls. Assuming an effect size of 0.8, an alpha probability of 0.05, an actual power of 0.8, and an allocation ratio (HD patients/ healthy controls) of 4, the required number of HD patients and healthy controls were 64 and 16 respectively. Subsequently, using clinical parameters and the lower-limb muscle rSO<sub>2</sub>, we performed a statistical power analysis in the multiple regression analysis. Assuming an effect size of 0.15, an alpha probability of 0.05, an actual power of 0.8, and the use of 2 predictors, 68 HD patients were required in this study. Therefore, we planned to include 68 HD patients and 16 healthy controls. The actual power of

this study was 0.79 for comparing the lower-limb muscle rSO<sub>2</sub> between HD patients and healthy controls, and 0.80 in the multiple regression analysis using clinical parameters and the lower-limb muscle rSO<sub>2</sub>. Therefore, our findings can be considered sufficiently reliable.

3) Were all subjects from the same dialysis unit?

**Response 3:**

This study was performed as a single-center observational study. We have added this description to the revised Materials and Methods section of our manuscript.

Page 5, Lines27-28:

“This was a single-center observational study and included HD patients who met the following criteria:”

4) Was there a power analysis? Participants:

**Response 4:**

We performed an initial statistical power analysis for the comparison of the lower-limb muscle rSO<sub>2</sub> between HD patients and healthy controls. Assuming an effect size of 0.8, an alpha probability of 0.05, an actual power of 0.8, and an allocation ratio (HD patients/ healthy controls) of 4, the required number of HD patients and healthy controls were 64 and 16 persons respectively. Subsequently, using clinical parameters and the lower-limb muscle rSO<sub>2</sub>, we performed a statistical power analysis in the multiple regression analysis. Assuming an effect size of 0.15, an alpha probability of 0.05, an actual power of 0.8, and using 2 predictors, 68 HD patients were required in this study. Therefore, we planned for this study to include 68 HD patients and 16 healthy controls. The actual power of this study was 0.79 for comparing the lower-limb muscle rSO<sub>2</sub> between HD patients and healthy controls, and 0.80 in the multiple regression analysis using clinical parameters and the lower-limb muscle rSO<sub>2</sub>. Therefore, our findings can be considered sufficiently reliable.

Define the following in the manuscript:

5) inclusion criteria, what is meant by unimpaired consciousness?

**Response 5:**

We changed the description of one of our inclusion criteria from “unimpaired consciousness” to “agreement for the purpose of this study” in Page 5, Line 29.

6) Exclusion criteria, what is meant by symptomatic ischemia of the lower? How was this determined?

**Response 6:**

Hemodialysis (HD) patients with these symptoms underwent angiography and/or magnetic resonance imaging to investigate for the presence of peripheral artery disease (PAD). Therefore, we excluded HD patients with symptomatic PAD in our study. We have revised the discussion section of our manuscript accordingly to include these details.

Page 10, Lines 14-26:

“HD patients with symptomatic lower-limb ischemia who were diagnosed with peripheral artery disease by angiography and/or magnetic resonance imaging were excluded from the study. The prevalence of subclinical peripheral artery disease in HD patients is approximately 20-25% [4]. We did not use methods such as the ankle-brachial pressure index to investigate the presence of peripheral artery disease in asymptomatic patients. Therefore, while we have excluded patients with symptomatic peripheral artery disease from the present study, we may have inadvertently included some with subclinical disease. In these patients, muscle rSO<sub>2</sub> might be reduced via the decrease in oxygen supply caused by the microcirculatory impairment due to subclinical peripheral artery disease. Thus, it would be necessary to perform arterial vascular examinations in future studies so that such patients can be excluded.”

Page 12, Lines 4-11:

“This study has several limitations. Firstly, its sample size was relatively small. Secondly, we could not evaluate the association between lower-limb muscle

rSO<sub>2</sub> and calculated nutritional markers, including normalized protein catabolic rate and body mass index, anthropometric measurements representing nutritional status, and the severity of protein-energy wasting. Finally, we did not routinely examine the arterial vascular status of HD patients without symptoms of peripheral artery disease. As such, it is possible that some HD patients with subclinical peripheral artery disease may have been included in this study.”

7) Was a physical exam done of the lower limb arterial circulation?

**Response 7:**

We routinely checked the degree of lower-limb edema present in all HD patients included in the study. However, we did not perform routine peripheral arterial examinations (such as confirming the presence of the femoral, popliteal, and dorsalis pedis pulses) because these patients were asymptomatic.

8) Were any type of arterial dopplers done on any of the subjects?

**Response 8:**

Unfortunately, we did not perform Doppler ultrasonography to investigate the arterial circulation of HD patients included in this study.

9) URR and Kt/V (clearance data) and other nutritional data (PCR) should be included in this analysis; these variables may affect the results.

**Response 9:**

As the reviewer has suggested, we have calculated the Kt/V and urea reduction ration in each patient and included these markers in the simple linear regression analysis for the lower-limb muscle rSO<sub>2</sub>. However, we did not find any significant associations between the lower-limb muscle rSO<sub>2</sub> and either of these two parameters. Unfortunately, other nutritional data including PCR could not be measured and calculated in this study. As such, future studies will be needed to confirm the associations between lower-limb muscle oxygenation and other nutritional markers. We have added these descriptions to the Materials and Methods section as well as Table 1.



Page 8, Lines 1-3:

“Furthermore, for evaluating the efficacy of HD, the Kt/V using the Daugirdas II formula <sup>[19]</sup> and the urea reduction ratio <sup>[20]</sup> were calculated in each patient.”

#### References:

19 **Daugirdas JT**. Second generation logarithmic estimates of single-pool variable volume Kt/V: An analysis of error. *J Am Soc Nephrol* 1993; **4**: 1205-1213 [PMID: 8305648]

20 **Owen WF Jr**, Lew NL, Liu Y, Lowrie EG, Lazarus JM. The urea reduction ratio and serum albumin concentration as predictors of mortality in patients undergoing hemodialysis. *N Engl J Med* 1993; **329**: 1001-1006 [PMID: 8366899 DOI: 10.1056/NEJM199309303291404]

10) Need to include more description of the dialysis treatment, what dialyzers etc., were used?

#### **Response 10:**

As suggested by the reviewer, we have added these descriptions to the revised Materials and Methods section.

Page 6, Lines 11-21:

“The HD dialysate comprised 140 mEq/L Na<sup>+</sup>, 2.0 mEq/L K<sup>+</sup>, 114.5 mEq/L Cl<sup>-</sup>, 2.5 mEq/L Ca<sup>2+</sup>, 1.0 mEq/L Mg<sup>2+</sup>, 25 mEq/L HCO<sub>3</sub><sup>-</sup>, and 150 mg/dL glucose. The dialyzer included polysulfone membrane (three patients; 1.0 m<sup>2</sup>, five patients; 1.3 m<sup>2</sup>, four patients; 1.5 m<sup>2</sup>, six patients; 1.6 m<sup>2</sup>, thirteen patients; 1.8 m<sup>2</sup>, nine patients; 2.0 m<sup>2</sup>, sixteen patients; 2.1 m<sup>2</sup>) and cellulose triacetate membrane (two patients; 1.3 m<sup>2</sup>, five patients; 1.5 m<sup>2</sup>). The dialysate flow rate in all patients was consistently 500 mL/min and the blood flow rate was 183 ± 1.7 mL/min in this study. The dialysate purification process followed the recommendations in the Japanese Society for Dialysis Therapy guidelines <sup>[15]</sup>.

The dialysate bacteria and endotoxin concentration in this study were less than 0.1 CFU/mL and 0.001 EU/mL respectively.”

Reference:

15 **Kawanishi H**, Akiba T, Masakane Y, et al.: Standard on microbiological management of fluids for hemodialysis and related therapies by the Japanese Society for Dialysis Therapy 2008. *Ther Apher Dial* 2009; **13**: 161-166 [PMID: 19379157 DOI: 10.1111/j.1744-9987.2009.00674.x]

11) Were the controls screened for any medical problems?

**Response 11:**

The healthy controls included in this study received annual physical examinations. None of them had any medical problems or took any medications.

12) I am concerned regarding the use of a blood sample from the arterial limb of the AVF. Did all subjects have an AVF? Did some have catheter or AVG access? This will affect the results.

**Response 12:**

Thank you for your comment. All HD patients included in this study had an arteriovenous fistula. We have added this to the revised Materials and Methods section.

Page 5, Lines 29-30:

“and (3) presence of arteriovenous fistula as a vascular access for HD.”

Results:

13) How do you interpret a negative correlation with duration of hemodialysis?

**Response 13:**

In this study, simple regression analysis demonstrated a significant negative correlation between the lower-limb muscle rSO<sub>2</sub> and the HD duration. However,

this correlation disappeared in the multiple linear regression analysis ( $p = 0.13$ ). Therefore, the association between the lower-limb muscle  $rSO_2$  and the HD duration was not considered to be significant in this study.

Discussion:

14) Overall very poorly written. First paragraph should summarize the main findings of the study. As written the first paragraph summarizes previous investigation by the authors. Would not make conclusions based on this study, it is only an observation.

**Response 14:**

As suggested by the reviewer, we have corrected the description in the revised Discussion section.

Page 9, Lines 3-14:

“In the present study, the lower-limb muscle  $rSO_2$  was significantly lower in HD patients than in healthy controls. Furthermore, multiple linear regression analysis indicated that serum inorganic phosphate and albumin concentrations, both of which represent nutritional status, were independently associated with the lower-limb muscle  $rSO_2$ .

Regarding the association between tissue oxygenation and HD therapy, cerebral  $rSO_2$  values were reported to be significantly lower in HD patients than in healthy controls [21,22]. These values were maintained during HD and not influenced by blood volume reduction [22], whereas intradialytic hypotension might be associated with a decrease in cerebral  $rSO_2$  [23]. Therefore, these findings indicate the importance of monitoring systemic tissue oxygenation, particularly cerebral oxygenation as a part of systemic oxygenation, in HD patients.”

*Reviewed by 00503199*

This is an interesting study. However clarification is needed.

- 1) Major concern is the distribution of the parameters studied as you have handled them as being all of them normally distributed. If this is the case, please provide this information in the text, otherwise you will have to redo the analysis using non-parametric tests and transformation of the non-normally distributed parameters.

**Response 1:**

Thank you for your comment. The Shapiro-Wilk test was used to confirm that all data were normally distributed. We have added these descriptions to the revised Materials and Methods section.

Page 8, Lines 7-8:

"The Shapiro-Wilk test was used to confirm that all data were normally distributed."

- 2) In addition, I wonder if you can examine at this stage the association of other parameters of malnutrition (BMI, nPCR etc) from the data that you have.

**Response 2:**

We appreciate your thoughtful comment. Unfortunately, other nutritional parameters including BMI and nPCR could not be measured and calculated in this study. As such, future studies will be needed to confirm the associations between lower-limb muscle oxygenation and other nutritional parameters.

- 3) Finally, I am not sure if the control group adds in this study. If I understand well it was not matched to the HD group (you have to provide the characteristics of the control group), thus I would consider delete it, as it is of limited and uncertain value. Other comments

**Response 3:**

We appreciate your thoughtful comment. As pointed out by the reviewer, there was no clinical information apart from the rSO<sub>2</sub> values because we were unable

to obtain clinical and blood gas parameters in the control group. However, the measurement of the lower-limb muscle rSO<sub>2</sub> has rarely been performed in patients with HD. As such, the measurement of rSO<sub>2</sub> before HD and the comparison of these values with the rSO<sub>2</sub> in the control group might be important. In this study, the relationship between the lower-limb muscle rSO<sub>2</sub> in patients with HD and in the controls was found to be consistent with that of previous reports. This confirms the accuracy of the rSO<sub>2</sub> measurement in this study; therefore, we mentioned the relationship between rSO<sub>2</sub> before HD and these values in the controls. We hope that our reply is acceptable.

- 4) Abstract: - Conclusion (also in main text): “was affected by” Please write ...was associated with.... Observational studies provide correlations not causations.

**Response 4:**

As suggested by the reviewer, we have corrected the description in the revised Abstract and Discussion sections.

Page 4, Line 8:

“In HD patients, the oxygenation of lower-limb muscle tissue was associated with serum inorganic phosphate and albumin concentrations, both of which represent nutritional status.”

Page 12, Line 16:

“Furthermore, the oxygenation of lower-limb muscle tissue was associated with serum inorganic phosphate and albumin concentrations, both of which represent nutritional status.”

- 5) Provide also a brief limitations' section in the abstract.

**Response 5:**

As suggested by the reviewer, we have added these descriptions to the Abstract section.

Page 3, Line 28-Page 4, Line 5:

**“LIMITATIONS:** This study has several limitations. First, the sample size of the present study was small. Second, we could not evaluate the association between lower-limb muscle rSO<sub>2</sub> and other nutritional markers such as normalized protein catabolic rate and body mass index. Finally, in included HD patients without symptoms of legs, we did not routinely perform the examination of arterial vascular studies; therefore, there were possibilities to include HD patients with subclinical peripheral artery disease in this study.”

- 6) Introduction: - Aims: “(2) clarify the mechanism influencing the value of lower-limb muscle oxygenation in HD patients” You examine associations, not mechanism

**Response 6:**

As suggested by the reviewer, we have corrected the description in the revised Introduction section.

Page 5, Line 22:

“(2) clarify the association influencing the value of lower-limb muscle oxygenation in HD patients.”

- 7) Methods: - Provide additional exclusion criteria, such as catheter as an access.

**Response 7:**

As suggested by the reviewer, we have added these descriptions to the Materials and Methods section.

Page 5, Lines 29-30:

“and (3) presence of arteriovenous fistula as a vascular access for HD.”

- 8) Was amputation an exclusion criterion? –

**Response 8:**

Thank you for your advice. As pointed out by the reviewer, we have added these descriptions to the Materials and Methods section.

Page 6, Lines 1-2:

“(2) symptomatic ischemia and receiving lower leg amputation.”

9) Measurement of rSO<sub>2</sub> in both legs? In the dominant one? Please explain.

**Response 9:**

Thank you for your comment. The lower-limb muscle rSO<sub>2</sub> was measured at the lateral side of the gastrocnemius muscle of the right lower-limb. All patients included in this study were right-handed, and the arteriovenous fistula was therefore situated on the left forearm. Therefore, the lower-limb muscle rSO<sub>2</sub> was measured at the right leg because this was opposite to the arteriovenous fistula. As suggested by the reviewer, we have added these descriptions to the Materials and Methods section.

Page 6, Line 30:

“Lower-limb muscle rSO<sub>2</sub> was monitored at right side lower-limb”

10) Control group: Provide site of arterial blood sampling Data analysis:

**Response 10:**

As mentioned above, there was no clinical information apart from the rSO<sub>2</sub> values because we were unable to obtain clinical and blood gas parameters in the control group. Therefore, we are unfortunately unable to provide the analysis of the arterial blood gas data in the control group.

11) Correlations between parameters (not groups). - Were all the parameters studied normally distributed? You have only used pearson's r, that is for normal distribution.

**Response 11:**

Thank you for your comment. All data were confirmed normally distributed by Shapiro-Wilk test. We have added these descriptions to revised Materials and Methods section.

Page 8, Lines 7-8:

“All data were confirmed normally distributed by Shapiro-Wilk test.”

12) Results: - provide characteristics of the control group

**Response 12:**

As mentioned above, we were only able to obtain data on age, sex, and lower-limb muscle rSO<sub>2</sub> values. Therefore, we regret that we cannot provide data on other characteristics in the control group.

13) Limitations: Add additional limitations: - You have not examined other indices of malnutrition – wasting: BMI, waist circumference, waist/hip circumference, biceps and triceps measurements, loss of body weight the last 6 months, normalized protein catabolic rate etc.

**Response 13:**

As advised by the reviewer, we have added these descriptions to the revised Discussion section.

Page 12, Lines 4-11:

“This study has several limitations. Firstly, its sample size was relatively small. Secondly, we could not evaluate the association between lower-limb muscle rSO<sub>2</sub> and calculated nutritional markers, including normalized protein catabolic rate and body mass index, anthropometric measurements representing nutritional status, and the severity of protein-energy wasting. Finally, we did not routinely examine the arterial vascular status of HD patients without symptoms of peripheral artery disease. As such, it is possible that HD patients with subclinical peripheral artery disease may have been included in this study.”

14) You have not examined the use of phosphate binders

**Response 14:**

As suggested by the reviewer, we have examined the use of phosphate binders and vitamin D metabolites in the HD patients included in the study. We have added these descriptions to the revised Materials and Methods section.



Page 6, Lines 9-11:

“Furthermore, the proportions of patients using phosphate binders and vitamin D metabolites were 71.6% (n = 48) and 37.3% (n = 25), respectively.”

15) The control group was not matched to the HD group

**Response 15:**

We completely agree with the reviewer’s comment. Unfortunately, we were unable to match the included HD patients and the healthy controls in this study. Future studies are necessary to compare the tissue oxygenation – including muscle rSO<sub>2</sub> – in matched HD patients and healthy controls.

16) You have not measured parameters of peripheral artery disease. (you have already mentioned it in a previous paragraph, but you can also remind it in the limitations section)

**Response 16:**

As advised by the reviewer, we have added these descriptions to the revised Discussion section.

Page 12, Lines 4-11:

“This study has several limitations. Firstly, its sample size was relatively small. Secondly, we could not evaluate the association between lower-limb muscle rSO<sub>2</sub> and calculated nutritional markers, including normalized protein catabolic rate and body mass index, anthropometric measurements representing nutritional status, and the severity of protein-energy wasting. Finally, we did not routinely examine the arterial vascular status of HD patients without symptoms of peripheral artery disease. As such, it is possible that some HD patients with subclinical peripheral artery disease may have been included in this study.”

17) Table 1. Provide p-values and r for all the parameters (not just ns).

**Response 17:**

As suggested by the reviewer, we have added these descriptions to Table 1.

18) You can have in bold those statistically significant if you like

**Response 18:**

As suggested by the reviewer, we have changed these styles in Table 1.

19) Table 2. Provide additional data as CI etc that are shown in the statistical package.

**Response 19:**

As advised by the reviewer, we have added these descriptions to Table 2.