PEER REVIEW HISTORY

ARTICLE DETAILS

TITLE (PROVISIONAL)	A protocol for a prospective observational study on the association of variables obtained by contrast-enhanced ultrasonography and sepsis-associated acute kidney injury
AUTHORS	liu, ning; Zhang, Zhongheng; Hong, Yucai; Li, Bing; Cai, Huabo; Zhao, Hui; Dai, Junru; Liu, Lian; Qian, Xin; Jin, Qicheng

VERSION 1 - REVIEW

REVIEWER	Kai M. Schmidt-Ott Charité - Universitätsmedizin Berlin Berlin, Germany
REVIEW RETURNED	21-May-2018

GENERAL COMMENTS	The study protocol “A prospective observational study protocol for examining the ability of contrast-enhanced ultrasound to predict sepsis-associated acute kidney injury“ introduces a prospective observational study in the ICU. Patients >18 yo with sepsis, severe sepsis or septic shock will be or have been enrolled on the day of ICU admission (between 08/11/2017 and 09/30/2019). Demographic information, chronic disease history, APACHE II score will be collected. Blood work, vital signs, CVP, SPO2, GCS, medication, intake and output, vasopressor requirement will be recorded during the following week after ICU admission. Patients will receive conventional duplex ultrasonography of the kidneys, heart, lungs, and IVC. Contrast enhanced ultrasonography (CEUS) will be performed to analyse renal microperfusion in the cortex and medulla. Transcutaneus oxygen pressure (TcPO2) will be used to detect cutaneous microperfusion. Approximately 200 patients will be enrolled. The authors plan to use ROC analyses to assess the ability of CEUS to predict AKI General assessment: The proposed prospective assessment of the ability of contrast-enhance ultrasound to predict sepsis-associated AKI is interesting and timely. However, the study protocol in its current form remains too imprecise with the regard to inclusion criteria, with regard to the definition of AKI, with regard to the timing of the AKI diagnosis relative to study inclusion/CEUS assessment, and with regard to outcomes. No primary outcome is defined nor is the planned analysis strategy described in sufficient detail. No power calculation is included to explain why the authors came up with the plan to enroll 200 patients. Overall, in its current form the study protocol is too superficial to enable the reader to understand and evaluate this study. Major concerns:

	1. How will the authors perform the analyses to see whether CEUS will predict sepsis-associated AKI? If the idea is to predict AKI, then it would be required that AKI is not yet present at the time of the ultrasound. What will be the detailed definition of AKI? At what time will the AKI diagnosis be made? Will patients with existing AKI at the time of ICU admission be included and how can CEUS be used to predict AKI in these patients if it is already present? How will baseline creatinine be determined to enable a reproducible AKI diagnosis? 2. Please indicate how many patients have already been enrolled in the study. 3. Definition of sepsis is “acute change of ≥2 points in the total Sequential Organ Failure Assessment score as a result of anti-inflammatory function. “ This sentence is confusing. I recommend restating to “…caused by a dysregulated host response to infection“. It would be helpful to provide some more detail on how the study physicians decide whether or not sepsis is present. 4. The authors mention that they will screen and enroll patients with severe sepsis. I recommend that they provide a definition for “severe sepsis” or remove the term “severe sepsis”. 5. The authors state: „We plan to recruit 200 patients, who will be divided into AKI and non-AKI groups based on their serum creatinine (SCr) levels. We will consider AKI to be present when blood urea nitrogen levels are >80 mg/dL or creatinine levels are >3 mg/dL.[10, 11].“ This is a very confusing statement. Will patients be divided into AKI and non-AKI subgroups at the time of inclusion? Will the relevant creatinine level be at the time of inclusion or during follow-up (and if the latter, during what duration of follow-up)? The AKI definition of BUN>80 or creatinine >3 is very unusual and does not concur with current consensus definitions of AKI. In the abstract, it is stated that the Acute Kidney Injury Network (AKIN) Criteria will be used, but this is not further specified in the methods section. It should be pointed out that according to current consensus guidelines the AKIN criteria have been replaced by the KDIGO criteria. 6. The authors conclude: „In conclusion, the present study aims to use CEUS to evaluate renal microcirculation changes in patients with and without AKI, which should provide information regarding whether CEUS can predict the risk of developing SA-AKI.“ This sentence illustrates the inherent imprecisions of the study in its current form: The study cannot evaluate the renal microcirculation changes in patients with AKI and at the same time predict the development of AKI.

REVIEWER	Luca Di Lullo Department of Nephrology and Dialysis, "L. Parodi - Delfino" Hospital, Colleferro (Rome), Italy
REVIEW RETURNED	02-Jul-2018

GENERAL COMMENTS	Dear Authors, I read with interest your paper concerning study protocol on CEUS indications in septic patients. I think that your protocol can be intersting to evaluate ultrasound aspects of septic patients. I have just a remark for you: in the Exclusion Criteria you have added patients with previous chronic kidney disease. I think that you should inckude CKD patients also

	according to disease's stage because stage 1 to Stage 3a patients could be included in the study protocol

REVIEWER	Nattachai Srisawat Chulalongkorn University
REVIEW RETURNED	17-Jul-2018

GENERAL COMMENTS	Comments to the authors The article “A prospective observation study protocol for examining the ability of contrast-enhanced ultrasound to predict sepsis-associated acute kidney injury” by Liu N, et al provide new knowledge in the field of sepsis-associated AKI. However, there are the specific points need to be addressed Major comments - In the Abstract part, the authors mention to use AKIN criteria for diagnosis AKI, but in the Method part, they mentioned to use serum creatinine at level of 3 mg/dL which is not the same as the AKIN criteria. Currently, KDIGO criteria has been widely accepted than AKIN criteria. Why the authors would like to use AKIN criteria or event cutoff point 3 mg/dL. Please clarify. - We do not use BUN as the marker of AKI in standard criteria (RIFLE 2004, AKIN 2007, KDIGO 2012) anymore. Why the authors still propose to use BUN value in the criteria? - The authors proposed to enroll 200 subjects, what is this number come from? - The authors plan perform CEUS at day 0, 1, 3, and 7. On day 0, Is this performed before resuscitation or after? If the patients present with AKI, are they excluded? The inclusion and exclusion criteria is not clear. - Does this study receive Ethical approval? - Please specify, how often the blood sample will be drawn? - Chronic kidney disease had 5 stages, which stage will be excluded? Please specify. - There are many parameters derived from CEUS. Which parameters will be the most important parameters? This will be very important for the primary objective of this study.

REVIEWER	Iwan C. C. van der Horst Department of Critical Care, University of Groningen, University Medical Center Groningen, The Netherlands
REVIEW RETURNED	23-Jul-2018

GENERAL COMMENTS	Remarks on bmjopen-2018-023981 Liu et al. A prospective observational study protocol for examining the ability of contrast-enhanced ultrasound to predict sepsis-associated acute kidney injury. Thank you for giving me the opportunity to review this manuscript on a novel protocol using ultrasonography to evaluate renal perfusion in patients with sepsis. I hereby present my remarks. Abstract: P3L11: ‘after sepsis’ is not really correct as the protocol is started as soon as the patient is diagnosed with sepsis. So ‘during’ or ‘with’ would be more acceptable. P3L18: ‘enrol’ should be ‘enroll’

	P3L23 & P3L33: please use either ‘ultrasonography’ or ‘ultrasound’. Not both. Strengths and limitations of this study: P4 L11/12: While ultrasonography is useful in the emergency setting, this protocol concerns a 7 day period. The statement that ‘this procedure’ can be perfomed and is useful in the emergency setting is therefore a strange one. Please change. P4L16: While inter-observer variability is indeed a limitation of the generalizability of the findings, this could be assessed in the protocol by perfoming additional analyses and see what influence it has. In addition, inter-operator variability is a variant of subjective differences, so this is mentioned twice in the same sentence. Please consider adding and changing this. Introduction: P5L11: please use more up-to-date references. When concerning sepsis, the sepsis-3 definition is used at the moment. (e.g. Singer M, Deutschman CS, Seymour CW, Shankar-Hari M, Annane D, Bauer M, Bellomo R, Bernard GR, Chiche JD, Coopersmith CM, Hotchkiss RS, Levy MM, Marshall JC, Martin GS, Opal SM, Rubenfeld GD, van der Poll T, Vincent JL, Angus DC (2016) The third international consensus definitions for sepsis and septic shock (Sepsis-3). JAMA 315:801–810. For sepsis definition. Fleischmann C, Scherag A, Adhikari NK, Hartog CS, Tsaganos T, Schlatt mann P, Angus DC, Reinhart K (2016) Assessment of global incidence and mortality of hospital-treated sepsis. Current estimates and limitations. Am J Respir Crit Care Med 193:259–272. For incidence and mortality.) P5L13: please use ‘mortality’ instead of ‘death’ P5L18/20: using both AKI and SA-AKI makes reading this manuscript confusing sometimes. Please consider only using one of these terms. P5L38: consider omitting the sentence concerning cardiovascular disease as risk factor as this manuscript is primarily about sepsis associated AKI. P5 L48: please add a reference concerning the recent research on the pathogenesis of SA-AKI. Methods and analysis: P6 L47: change the sentence “all patients will be educated”. Most of the patients will be ventilated and sedated so they are unable to be educated. P7L4: change ‘stay of <24h’ to ‘an expected stay of <24h’. In addition, why is a DNR order chosen as exclusion criterion? P8L4: please add a table or figure or the work sheet showing the data that will be collected. P9L21: please add references to the protocols of echocardiography and lung ultrasonography. E.g. the BLUE protocol where B-lines are described and explained. P11L8: please describe how the Transcutaneous oxygen pressure (TcPO2) is monitored. How will this be evaluated statistically? Discussion: The first part of the discussion is focused on the role of CRRT in AKI, this however is not the scope of the manuscript so should be omitted. Please focus on AKI in sepsis and why assessing it using ultrasonography is necessary. P12L11: stating CRRT is a useful method for managing AKI does not seem correct. CRRT is used as a supportive treatment as bridge to recovery and is not a managing tool. Please change.

	P15L40: if the long interval between CEUS examinations is a limitation which may delay the diagnosis in patients who develop SA-AKI, why was this long interval chosen?

VERSION 1 – AUTHOR RESPONSE

Reviewer: 1

Major concerns:

1. How will the authors perform the analyses to see whether CEUS will predict sepsis-associated AKI? If the idea is to predict AKI, then it would be required that AKI is not yet present at the time of the ultrasound. What will be the detailed definition of AKI? At what time will the AKI diagnosis be made? Will patients with existing AKI at the time of ICU admission be included and how can CEUS be used to predict AKI in these patients if it is already present? How will baseline creatinine be determined to enable a reproducible AKI diagnosis?

Reply: thank you very much for the comments. In our study, we would separate patients into four categories (no SA-AKI, stage 1, stage 2, stage 3 SA-AKI) after 7 consecutive days of monitoring and then try to detect the changes of microcirculation in cortex and medulla of the kidney by using CEUS parameters including baseline intensity, arrival time, time-to-peak, peak intensity, ascending slope,descending time/2, descending slope, and area under the curve . Therefore, we need to make sure that AKI is not yet present at the time of the ultrasound. Patients with existing AKI at the time of ICU admission will be excluded. In our study, SA-AKI were diagnosed by KDIGO criteria.Baseline creatinine value (μmol/L) was either registered from 6 months previous clinical files or estimated, when data was not available from clinical records, by solving the Modification of Diet in Renal Disease (MDRD) equation assuming a glomerular filtration rate of 75 ml/min/1.73 m2 .

2. Please indicate how many patients have already been enrolled in the study.

Reply: Actually, there were only 15 patients enrolled in the study at present.

3. Definition of sepsis is “acute change of ≥2 points in the total Sequential Organ Failure Assessment score as a result of anti-inflammatory function. “ This sentence is confusing. I recommend restating to “…caused by a dysregulated host response to infection“.It would be helpful to provide some more detail on how the study physicians decide whether or not sepsis is present.

Reply: we modified this sentence according to the comments.

4. The authors mention that they will screen and enroll patients with severe sepsis. I recommend that they provide a definition for “severe sepsis” or remove the term “severe sepsis”.

Reply: we remove the term“severe sepsis” according to “the Third International Consensus Definitions for Sepsis and Septic Shock (Sepsis-3)”.

5. The authors state: We plan to recruit 200 patients, who will be divided into AKI and non-AKI groups based on their serum creatinine (SCr) levels. We will consider AKI to be present when blood urea nitrogen levels are >80 mg/dL or creatinine levels are >3 mg/dL.[10, 11].“ This is a very confusing statement. Will patients be divided into AKI and non-AKI subgroups at the time of inclusion? Will the relevant creatinine level be at the time of inclusion or during follow-up (and if the latter, during what duration of follow-up)? The AKI definition of BUN>80 or creatinine >3 is very unusual and does not concur with current consensus definitions of AKI. In the abstract, it is stated that the Acute Kidney Injury Network (AKIN) Criteria will be used, but this is not further specified in the methods section. It should be pointed out that according to current consensus guidelines the AKIN criteria have been replaced by the KDIGO criteria.

Reply: The current definition by Kidney Disease Improving Global Outcomes (KDIGO) is similar to the AKIN definition, but the time frame is extended from 48 hours to 7 days. The KDIGO criteria evaluate baseline SCr and, therefore, can detect AKI in patients with slow increases in SCr. In the revision, we modified this sentence as " In 7 days later, the total of septic patients were stratified into AKI (including stage 1, 2, and 3) and non-AKI groups according to Kidney Disease Improving Global Outcomes (KDIGO) criteria for further statistical analysis.Patients with sepsis were enrolled and followed up for the occurrence of AKI. They were not categorized into AKI and non-AKI group at the beginning, but AkI is an outcome during our study period.

6.The authors conclude: „In conclusion, the present study aims to use CEUS to evaluate renal microcirculation changes in patients with and without AKI, which should provide information regarding whether CEUS can predict the risk of developing SA-AKI." This sentence illustrates the inherent imprecisions of the study in its current form: The study cannot evaluate the renal microcirculation changes in patients with AKI and at the same time predict the development of AKI.

Reply: we clarify in the revision that similar studies have been carried out in which the perfusion of the renal cortex is significantly reduced in patients with severe AKI occurrence (KDIGO stages 2 or 3) in course of sepsis shock by using CEUS. However, they only measured cortical perfusion in patients with septic shock while medullary perfusion was not mentioned. In our study, we try to find the correlation between the change of microcirculation in kidney and the occurrence or progression of SA-AKI .

Reviewer: 2

I read with interest your paper concerning study protocol on CEUS indications in septic patients.

I think that your protocol can be intersting to evaluate ultrasound aspects of septic patients. I have just a remark for you: in the Exclusion Criteria you have added patients with previous chronic kidney disease. I think that you should inckude CKD patients also according to disease's stage because stage 1 to Stage 3a patients could be included in the study protocol

Reply: Thank you very much for the comments. Yes, stage 1 to Stage 3a patients could be included in the study protocol. We clarified this in the revision as"The presence of end-stage chronic kidney disease or long-term haemodialysis should be excluded ."

Reviewer: 3

Major comments

- In the Abstract part, the authors mention to use AKIN criteria for diagnosis AKI, but in the Method part, they mentioned to use serum creatinine at level of 3 mg/dL which is not the same as the AKIN criteria. Currently, KDIGO criteria has been widely accepted than AKIN criteria. Why the authors would like to use AKIN criteria or event cutoff point 3 mg/dL. Please clarify.

Reply: thank you very much for the comments. I regret to say that it is my problem to confuse the two definitions.

The current definition by Kidney Disease Improving Global Outcomes (KDIGO) is similar to the AKIN definition, but the time frame is extended from 48 hours to 7 days. The KDIGO criteria evaluate baseline SCr and, therefore, can detect AKI in patients with slow increases in SCr. In the revision, SA-KI was diagnosed according to Kidney Disease Improving Global Outcomes (KDIGO) criteria .

- We do not use BUN as the marker of AKI in standard criteria (RIFLE 2004, AKIN 2007, KDIGO 2012) anymore. Why the authors still propose to use BUN value in the criteria?

Reply: In the revision, SK-AKI was diagnosed according to Kidney Disease Improving Global Outcomes (KDIGO) criteria according to changes in creatinine and urine output.

- The authors proposed to enroll 200 subjects, what is this number come from?

Reply:we assumed that the diagnostic performance of CEUS parameter has the AUC of 0.85 for a particular test is significant from the null hypothesis value 0.5 (meaning no discriminating power). We expect to include twice as many negative cases than positive cases, so for the Ratio of sample sizes in negative / positive groups you enter 2. For α-level we select 0.05 and for β-level you select 0.20 (power is 80%). Also to account for loss to follow up, the sample size was 200.

- The authors plan perform CEUS at day 0, 1, 3, and 7. On day 0, Is this performed before resuscitation or after? If the patients present with AKI, are they excluded? The inclusion and exclusion criteria is not clear.

Reply: On day 0, for septic shock patients, we performed CEUS before fluid resuscitation, and then we would re-evaluate renal microvascular alterations by the use of CEUS with resuscitation targeted at normalization of blood pressure. Patients with existing AKI at the time of ICU admission would not be excluded, because we also want to monitor the microcirculation perfusion of kidney in different stages of SA-AKI.We clarified this in the revision.

- Does this study receive Ethical approval?

Reply: Yes, The study protocol was approved by the ethics committee of Sir Run Run Shaw Hospital (Zhejiang University Medical College,registration number: 2016C91401).

- Please specify, how often the blood sample will be drawn?

Reply: We clarified this in the revision as“the frequency of laboratory tests in patients with stable vital signs is once a day, however, for septic shock patients, laboratory tests may be performed every 8 hours.”

- Chronic kidney disease had 5 stages, which stage will be excluded? Please specify.

Reply: Serious changes in the structure of the kidney with end stage renal disease or long-term haemodialysis are not conducive to the monitoring of microcirculatory perfusion in our study. We have clarified this in the revision.

- There are many parameters derived from CEUS. Which parameters will be the most important parameters? This will be very important for the primary objective of this study.

Reply: The slope rate of the cortical ascending curve , the medullary ascending curve , and the peak intensity have been proved different between patients with normal and abnormal SCr levels by using CEUS. A larger cohort of patients in our study would have allowed us to certify if increasing severity of AKI is associated with the changes of these parameters.

Reviewer: 4

Abstract:

P3L11: ‘after sepsis’ is not really correct as the protocol is started as soon as the patient is diagnosed with sepsis. So ‘during’ or ‘with’ would be more acceptable.

Reply: thank you very much for the comments. we modified this word according to the comments.

P3L18: ‘enrol’ should be ‘enroll’

Reply:we modified this word in the revision.

P3L23 & P3L33: please use either ‘ultrasonography’ or ‘ultrasound’. Not both.

Reply:we used the word ‘ultrasonography’ in the revision.

Strengths and limitations of this study:

P4 L11/12: While ultrasonography is useful in the emergency setting, this protocol concerns a 7 day period. The statement that ‘this procedure’ can be perfomed and is useful in the emergency setting is therefore a strange one. Please change.

Reply:We have clarified this in the revision as" This uncomplicated and non-nephrotoxic procedure with the ability to detect subtle perfusion abnormalities quickly and in real-time has advantages over traditional modes , which is especially useful in the emergency setting.".

P4L16: While inter-observer variability is indeed a limitation of the generalizability of the findings, this could be assessed in the protocol by perfoming additional analyses and see what influence it has. In addition, inter-operator variability is a variant of subjective differences, so this is mentioned twice in the same sentence. Please consider adding and changing this.

Reply:We have clarified this in the revision as " Subjective differences and patient heterogeneity may limit the generalizability of the findings.".

Introduction:

P5L11: please use more up-to-date references. When concerning sepsis, the sepsis-3 definition is used at the moment. (e.g. Singer M, Deutschman CS, Seymour CW, Shankar-Hari M, Annane D, Bauer M, Bellomo R, Bernard GR, Chiche JD, Coopersmith CM, Hotchkiss RS, Levy MM, Marshall JC, Martin GS, Opal SM, Rubenfeld GD, van der Poll T, Vincent JL, Angus DC (2016) The third international consensus definitions for sepsis and septic shock (Sepsis-3). JAMA 315:801–810. For sepsis definition. Fleischmann C, Scherag A, Adhikari NK, Hartog CS, Tsaganos T, Schlatt mann P, Angus DC, Reinhart K (2016) Assessment of global incidence and mortality of hospital-treated sepsis. Current estimates and limitations. Am J Respir Crit Care Med 193:259–272. For incidence and mortality.)

Reply: we modified this sentence according to the comments.

P5L13: please use ‘mortality’ instead of ‘death’

Reply:we modified this word according to the comments.

P5L18/20: using both AKI and SA-AKI makes reading this manuscript confusing sometimes. Please consider only using one of these terms.

Reply:we used the word ‘SA-AKI’ in the revision.

P5L38: consider omitting the sentence concerning cardiovascular disease as risk factor as this manuscript is primarily about sepsis associated AKI.

Reply: we omitted this sentence according to the comments.

P5 L48: please add a reference concerning the recent research on the pathogenesis of SA-AKI.

Reply: we modified this sentence according to the comments.

Methods and analysis:

P6 L47: change the sentence “all patients will be educated”. Most of the patients will be ventilated and sedated so they are unable to be educated.

Reply: we modified this sentence according to the comments.

P7L4: change ‘stay of <24h’ to ‘an expected stay of <24h’. In addition, why is a DNR order chosen as exclusion criterion?

Reply:Yes, DNR patients are not strictly excluded. We modified this in the revision.

P8L4: please add a table or figure or the work sheet showing the data that will be collected.

Reply:Yes, we will upload a work sheet.

P9L21: please add references to the protocols of echocardiography and lung ultrasonography. E.g. the BLUE protocol where B-lines are described and explained.

Reply: Two new references were as followed.

1.Lichtenstein DA. BLUE-protocol and FALLS-protocol: two applications of lung ultrasound in the critically ill. Chest 2015;147:1659-70.

2.Perera P, Mailhot T, Riley D, et al. The RUSH exam: Rapid Ultrasound in SHock in the evaluation of the critically lll. Emergency medicine clinics of North America 2010;28:29-56.

P11L8: please describe how the Transcutaneous oxygen pressure (TcPO2) is monitored. How will this be evaluated statistically?

Reply:The aim of the protocol is to study renal microcirculation perfusion by contrast-enhanced ultrasound. Therefore, so I deleted some research about TcPO2.

Discussion:

The first part of the discussion is focused on the role of CRRT in AKI, this however is not the scope of the manuscript so should be omitted. Please focus on AKI in sepsis and why assessing it using ultrasonography is necessary.

Reply: we modified this sentence according to the comments.

P12L11: stating CRRT is a useful method for managing AKI does not seem correct. CRRT is used as a supportive treatment as bridge to recovery and is not a managing tool. Please change.

Reply: Managing AKI requires two-pronged management: source control of sepsis (eg.drainage of the obstructive biliary system and antibiotics), accompanied in parallel by intensive monitoring and multiorgan support in the ICU. The role of CRRT in AKI was not the core content of the manuscript, so I deleted it.

P15L40: If the long interval between CEUS examinations is a limitation which may delay the diagnosis in patients who develop SA-AKI, why was this long interval chosen?

Reply:The purpose of the study is to examine the diagnostic performance of CEUS, thus early prediction is of clinical importance that it allows time to early prevention of potential kidney injury. If the prediction is made very close to the occurrence of AKI, then there will not be enough time for intervention. The aim is to predict AKI as early as possible.

VERSION 2 – REVIEW

REVIEWER	Kai Schmidt-Ott Charité - Universitätsmedizin Berlin Germany
REVIEW RETURNED	13-Jan-2019

GENERAL COMMENTS	Please insert details on ethics approval and study registration.

REVIEWER	Nattachai Srisawat Chulalongkorn University, Bangkok, Thailand
REVIEW RETURNED	28-Oct-2018

GENERAL COMMENTS	The authors responded to all of my questions. I do not have any questions

REVIEWER	Iwan C.C. van der Horst University of Groningen, University Medical Center Groningen, The Netherlands
REVIEW RETURNED	10-Nov-2018

GENERAL COMMENTS	A study on kidney perfusion over time in patient with sepsis to gain insight in acute kidney injury is of great value. The design including serial measurements is great. I think the fully appreciate the study more attention should be paid on the current manuscript. The authors make many statements and seem not supported within the manuscript. Please consider to focus on what contrast enhanced ultrasound has to offer to unravel underlying causes of acute kidney injury in sepsis. The main comment is on the statement contrast enhanced ultrasound gives information for predicting sepsis-associated acute kidney injury risk. First, how does a diagnostic predict risk? Second, what is the definition of sepsis-associated acute kidney injury?

	To guide the authors in clarifying statements I have several comments/suggestions in order of the text. Language: - The standard of written English needs work. Title: - A study protocol might not be prospective. Please consider to alter the title to: A protocol for a prospective observational study... - Is this a study to examen the ability or is the study on the association of variables obtained by contrast enhanced ultrasound and acute kidney injury? Abstract: - Acute kidney injury is often observed in patients with sepsis. In these patients acute kidney injury can result from various underlying causes. Why did the authors define acute kidney injury as sepsis-associated? Please consider to just state acute kidney injury in patients with sepsis. - Is it to predict the risk? I think it is to predict the development. I think many patients with sepsis will be at risk and contrast enhanced ultrasound might be associated with risk. - What is conventional ultrasonography? - In 7 days later? - The scans data? - Please explain what is time-intensity curves that characterize renal microcirculation. - What are blood volume and velocity parameters and why are they obtained/measured? - Datas will be compared? Why? What is expected? Or just multiple associations/correlations? What is considered significant? - In general, I think the methods should be more on the hypothesis, the primary outcome measures and should explain how and why measures are obtained. Strengths/limitations: - Is it a strength being the first? I think the topic is of great interest irrespective of being first. - Please explain in detail strength number 2. I think detecting abnormalities with contrast enhanced ultrasound is not equal to being useful. - What are subjective differences? - Please explain why patient heterogeneity in this study is especially a limitation? I think heterogeneity is hampering research in critically ill patients in general and not specifically in contrast enhanced ultrasound. It even might be speculated that performing contrast enhanced ultrasound might limit heterogeneity by observing similarities between clusters of patients. Introduction: - Please consider not to summarize all potential causes of acute kidney injury. I consider focusing on ultrasound in patients with acute kidney injury. - Is one reference sufficient to state that sepsis is the driver for acute kidney injury? And is one reference sufficient to state 50%? - Please present references when stating many studies revealed. - When stating the pathophysiology of acute kidney injury in sepsis differs from acute kidney injury in other diseases why did the

	authors choose to only include patients with sepsis in the current study? - Is epidemiological data able to show causal relationships? - Please present at least one reference for the statement: recent attention ... microcirculation alterations. - What is the definition of during and after sepsis. When is a patient no longer septic? If a study focuses on alterations in sepsis a clear definition for after sepsis seems necessary. - Does contrast identify blood flow to an organ or is it contrast (perfusion) through an organ? - What is meant by stating is useful for evaluating microcirculation perfusion in pancreatic, ..? - Is the primary objective to use (?) to evaluate? I think contrast enhanced ultrasound can be used and it might not be an objective to use but to evaluate .... - Expected to propose? - Can a single study provide gold standard parameters? - Do the authors suggest that stage 0 to 4 are to be considered as increasing disorder of renal perfusion? I think the authors stated earlier that acute kidney injury is heterogenous and I think perfusion disorders might result from underlying causes and on the other hand might be the cause for developing or worsening renal failure. Please consider to rephrase both aims. Methods: - Please speculate on missing patients in septic shock. I think more severely ill patients are unable to be educated. - How do the authors identify patients with obstruction? - Does it take 2 years to include 200 patients? - Are 200 patients sufficient for making strong conclusions? Did the authors compute a power for a primary outcome measure? - The critical care ultrasound protocol should be presented in detail. Furthermore, definitions for cut-offs should be presented. - Is an independent (core-laboratory) specialist involved in image quality? - How is data stored? - Are caregivers blinded for the information? - Is there a time limit for study diagnostics? Statistics - Statistics on serial measurements with multiple variables is very difficult. The current description of the statistics is not sufficient to understand the statistical methods that will be used. - Does a statistical analysis plan exist and which analyses will be performed? - Is a p-value of 0.05 sufficient for multiple testing? Discussion: - In general, try to focus on ultrasonography and kidney failure and not on general information on sepsis. - The discussion is to my opinion too long. - Discrepancy exist between numbers used in the introduction and the discussion. - How is acute kidney injury manageable? - Why discuss biomarkers? - When referring to studies on perfusion please present more details. What is the perfusion of the renal cortex is significantly reduced? To what percent? - Please consider to present the information on the contrast agent within the methods.

	- The conclusion should be equal throughout the manuscript and should answer the research question/primary objective.

VERSION 2 – AUTHOR RESPONSE

2.Reply to Reviewer 4 :

Thank you very much for that you have recommended detailed comments to my revisions. The opinions you provided are very meticulous and helpful. We have retrieved a large number of the latest references about contrast-enhanced ultrasonography and the pathobiology of sepsis-associated acute kidney injury. Then, we revised the draft very carefully in the revision.

Reply to Reviewer 1:

Thank you very much for the comments. we have added some details on ethics approval and study registration as " Ethics and dissemination: The study protocol was approved on 2 August 2017 by the Ethics Committee of Sir Run Run Shaw Hospital (Zhejiang University Medical College) ,(approval numbe: 2016C91401). The results will be published in a peer-reviewed journal and shared with the worldwide medical community within 2 years after the start of the recruitment. Trial registration: ISRCTN 14728986; Pre-results".

VERSION 3 - REVIEW

REVIEWER	Iwan van der Horst University of Groningen, University Medical Center Groningen, The Netherlands
REVIEW RETURNED	21-Mar-2019

GENERAL COMMENTS	With interest I read the revised version of the manuscript. To my opinion it is much more focused and clear.