PEER REVIEW HISTORY

BMJ Open publishes all reviews undertaken for accepted manuscripts. Reviewers are asked to complete a checklist review form (**http://bmjopen.bmj.com/site/about/resources/checklist.pdf**) and are provided with free text boxes to elaborate on their assessment. These free text comments are reproduced below.

ARTICLE DETAILS

TITLE (PROVISIONAL)	The Effect of Combined Testing of Ceramides with High-sensitive Troponin T on the Detection of Acute Coronary Syndrome in Patients with Chest Pain in China：a prospective observational study
AUTHORS	Yao, Kang; Wang, Yanzhong; Liu, Xuebo; shen, chengxing; Hu, Wei; Wang, Zhe; Wu, Runda; Tang, Xianglin; Sun, Aijun; Zou, Yunzeng; Qian, Juying; Wu, Guangyu; Guo, Xin; Cheng, Xiaoliang; Ge, Junbo

VERSION 1 - REVIEW

REVIEWER	Professor Paul Collinson Clinical Blood Sciences and Cardiology, St George's University Hospitals NHS Foundation Trust and St George's University of London, UK
REVIEW RETURNED	18-Dec-2018

GENERAL COMMENTS	The effect of combined testing of ceramides with high sensitive troponin C on the detection of ACS in patients with chest pain in China: a cross sectional study. The authors have undertaken a prospective observational study to assess the additional value of the measurement of ceramides for this prediction in patients with acute coronary syndrome (ACS) in a Chinese population. They demonstrate that addition of specific ceramides may improve diagnostic deficiency. A significant problem with this manuscript is a lack of the relevant analytical information which is essential for a study such as the one described. The authors need to address the following points in the manuscript. 1. The authors use the term ACS in the title without declaring this as acute coronary syndrome. 2. Page 5 (introduction) second paragraph, line 3. Measurement of creatine kinase MB isoenzyme is no longer considered as a sensitive and specific marker of myocardial injury. Troponin alone is used. This statement should therefore be modified. 3. Page 5 (introduction) line 3. “However, lesion low-density cholesterol etc”. What are the authors referring to in this statement. I presume they are referring to lipid content of atherosclerotic plaque. Please will they clarify this statement.

	4. A cursory search of the literature reveals 4 other papers that examine ceramides and ACS. The authors mention only one of these in the discussion section. A statement that this is the first paper seems somewhat inappropriate. These references should also be incorporated both in the introduction and the discussion. 1. de Carvalho LP, Tan SH, Ow GS, Tang Z, Ching J, Kovalik JP et al. Plasma Ceramides as Prognostic Biomarkers and Their Arterial and Myocardial Tissue Correlates in Acute Myocardial Infarction. JACC Basic Transl Sci 2018; 3:163-175. 2. Anroedh S, Hilvo M, Akkerhuis KM, Kauhanen D, Koistinen K, Oemrawsingh R et al. Plasma concentrations of molecular lipid species predict long-term clinical outcome in coronary artery disease patients. J Lipid Res 2018; 59:1729-1737. 3. Laaksonen R, Ekroos K, Sysi-Aho M, Hilvo M, Vihervaara T, Kauhanen D et al. Plasma ceramides predict cardiovascular death in patients with stable coronary artery disease and acute coronary syndromes beyond LDL-cholesterol. Eur Heart J 2016; 37:1967-1976. 5. Page 7. Laboratory methods. The authors need to supply the following information. What samples were taken for laboratory analyses (whole blood, serum or plasma), what sample tubes were used, the preparation and storage of samples prior to analysis. Duration of sample storage. In addition, for each of the analytes used in the study the authors need to supply significantly more information on the laboratory methodology. This can be supplied as a supplementary appendix but should include a brief overview of methodology (excluding abbreviations which will not be clear to the reader) detection limit, analytical range, imprecision profile and reference interval or decision limit as appropriate. In particular, the authors need to state for the cardiac Troponin T assays the 10% imprecision and 99th percentile reference interval used for diagnosis. In addition, if a male and female reference limits was used as is now recommended. 6. Page 7. Diagnosis of ACS. The diagnosis of myocardial infarction should be made using the international definition of myocardial infarction version 3, ideally version 4 in addition to American Heart Association (not abbreviated) guidelines. There is also a spelling error in the penultimate line “cardia” for cardiac. 7. Page 8. Ceramide methodology. Blood sampling needs to be specified (see above). Were samples centrifuged prior to analysis? Instrumentation parameters should be included in the data appendix should the dilution protocol. 8. Page 13 and 14. The authors comment on the potential for improving diagnostic classification. However, I do not get a feeling for whether or not the measurement of ceramides is applicable in the routine clinical laboratory and the authors should make some comment on the feasibility. Certainly, I do not see this test being delivered in real time with a 60 minute turnaround.

REVIEWER	Arash Mokhtari Department of Emergency Medicine and Internal Medicine, and Department of Cardiology, Lund University, Skane University Hospital in Lund Lund, Sweden
REVIEW RETURNED	21-Jan-2019

GENERAL COMMENTS	In this study by Yao et al. the authors aimed to evaluate the value of ceramides in the diagnosis of ACS in chest pain patients. The study population was chest pain patients, their index test was ceramide levels and their outcome a final diagnosis of ACS as adjudicated by 2 independent cardiologists. What the authors found was that (Cer(d18:1/24:1(15Z))/Cer(d18:1/24:0)) ratio, Cer(d18:1/14:0) and Cer(d18:1/22:0) were independent predictors of ACS. They then conclude that ceramides are of diagnostic value for detecting ACS. This is a large multicenter study, and the premise is interesting. I do however have several concerns stated below: Major: 1.The “study design and participants” section needs clarification. 1a: What this a planned analysis, or is this a secondary analysis of a study performed for another purpose? 1b: You state that you included chest pain patients who were admitted. Where they included after admission to a ward (and in that case, which wards?) or are you referring to ED admission? Who included patients, authors? Research nurses? During what time of day and which days of the week? Did you include patients 24/7 all days of the week? 1c: Why did you choose these specific exclusion criteria which are somewhat unusual for a chest pain study? You also state that patients with chronic kidney disease were excluded but what GFR did you use as cut-off for exclusion? You also have “other conditions” as an exclusion criteria, what exactly was included in this definition? 1d. Why did you include patients with STEMI? You state that your aim was to evaluate the value of ceramides for diagnosing ACS, but we do not rely on biomarkers for diagnosing patients with STEMI, which is a diagnosis based on the ECG. I would recommend excluding patients with STEMI as to be able to evaluate your biomarkers in the population you aim for it to be used clinically. 1e: I would like you to make a flowchart to include in the manuscript, and that you in the initial results section report how many who were screened for eligibility and how many who were excluded (reported for each exclusion criteria). This is part of the STROBE checklist (point 13), where you state that this is reported on p10, but it is not. 1f: Where there any missing data? Did all patients have blood samples stored? If not, what happened to patients who did not, where they excluded (not stated as exclusion criteria)? 2. The “Diagnosis of ACS” section also needs further clarification. 2a: The diagnosis of ACS was based on adjudication by 2 independent cardiologists. But what did you do with the cases where the 2 cardiologists disagreed? Please also report the agreement level and kappa value between the adjudicators in the results section. 2b. Was ceramide levels available to the adjudicators or were they blinded to the index test? 2c. You specifiy that “Cardiac chest pain and elevations in troponins levels without ST elevation indicate NSTEMI.” The diagnosis of MI should however also be based on a significant rise/fall of troponin levels as well, as defined in the universal MI definition criteria (Thygesen et al. Fourth Universal Definition of Myocardial Infarction. 2018). Was serial troponin levels also part of the definition criteria your

	adjudicators used? If so, please report how a significant change in serial hs-cTnT was defined at different troponin time samplings. 2d: Did you include both type 1 and type 2 MI or only type 1? 3. How was sample size determined? In the STROBE checklist point 10 you state that this is described in p7, but it is not. 4. You state that your aim was to "assess the value of ceramides in detection of ACS in patients with chest pain". In clinical practice we are interested in knowing which patients have a low enough risk of having ACS and who can be discharged (ruled-out), and which patients who have a high enough risk and who should be admitted and for example undergo coronary angiography (ruled-in). With regards to rule-out, achieving this for MI is rather straight forward. We evaluate the ECG to exclude STEMI and we can thereafter use only the admission (0h) hs-cTnT in some lower risk patients and in most other chest pain patients we can use a 0h in combination with a second hs-cTnT after 1-3h and achieve a very high negative predictive value for MI. This is also the approach recommended by the European Society of Cardiology (Roffi et al. ESC guidelines NSTE-ACS 2015). If you want to show the added value of ceramides in this situation, you need to for example show that a combination of ceramides + hs-cTnT enables us to identify more patients for rule-out using only 0h testing (increased efficiency), or that adding ceramides to hs-cTnT testing yields an increase in sensitivity and negative predictive value (increased safety). I do not believe that you have shown this in this study. This approach would require calculating a cut-off to be used in clinical practice for the different ceramides. If you could do this, and present diagnostic accuracy measures (sensitivity, specificity, predictive values, likelihood ratios) for ceramides and hs-cTnT and when combined, this would better answer your aim of the study and provide data that I believe is more clinically applicable. This way I can as a reader easier draw conclusions regarding the potential clinical implications. The primary difficulty of rule-out/rule-in is among patients with slightly elevated hs-cTnT and without a significant change on serial sampling. You state yourselves in the discussion that "However, high-sensitive troponin assays identify a larger number of patients with elevated troponin results but without a final diagnosis of ACS, making interpretation of test results challenging" which is a great point. But you have not shown that the addition of ceramides provides some added value in this context either, which also would require going about it as stated above for rule-out. Finally, even though ruling out MI is rather straight forward in most cases, the diagnosis of UA is not. It would be interesting to see if ceramides could improve upon the diagnosis of UA, where we know that hs-cTnT by itself is clearly inadequate. This would again require calculating diagnostic accuracy measures and evaluating the potential added value. 5. I may have misinterpreted this, but as I understand it Qlabs is a medical company that helped you with the analysis and interpretation of data. Several of the co-authors also seem to work for this company. Yet you state that there are no conflicts of interest. Does this company provide this analysis, and if so, I would believe that this is a clear conflict of interest. Minor:

	7. 12,8% of patients were diagnosed with ACS, how many were STEMI, NSTEMI and UA respectively? 8. How many of your patients' hade serial troponin measurements? At what time intervals did you measure serial hs-cTnT in these hospitals? Did all patients have hs-cTnT measurements? 9. Among those defined as having UA, how many had objective findings such as ECG changes, pathological objective testing such as SPECT, CCTA, or a significant stenosis on coronary angiography? 10. I would recommend re-phrasing the following in the discussion: 10a: "Ceramides measurement in high throughput quality-controlled environments is straightforward and cost-efficient". I do not believe you have shown in this study that measuring ceramide levels in chest pain patients is cost efficient. 10b. "By setting up clinical laboratories equipped with robotized sample handling systems and mass spectrometry equipment, it would be feasible and practical to identify patients with chest pain at high cardiovascular risk using our ceramides-based diagnostic model." I do not feel that you have shown why this would be practical (see also point 4 under "Major"). 10c. "However, with our model, ACS risk evaluation can be improved with high precision, thereby avoiding delays in treatment for those at high risk and also avoiding invasive, lengthy and expensive tests for those at low risk." I do not believe you have shown in this study that you could actually improve upon ACS diagnosis with ceramides (see point 4), nor that it would avoid delays or further testing. 11. In the limitations section please include a discussion on issues of generalizability, the fact that your results would need validation in other cohorts, as well as a discussion on other sources of potential bias such as selection bias. 12. I recommend some changes for the tables: 12a: There are too many decimals in several places 12b: Abbreviations should be explained below each table 12c: In table 1 please include more clinical data such as how many underwent further testing with echocardiography, coronary angiography or non-invasive testing (such as SPECT, CCTA, exercise ECG). 12d: The hs-cTnT levels presented in table 1 are ng/L and should therefore be corrected (i.e. not 0.009 but instead 9). 13. I feel the STARD checklist would be more appropriate than the STROBE checklist for this study

VERSION 1 – AUTHOR RESPONSE

Reviewer: 1
Reviewer Name: Professor Paul Collinson

Institution and Country: Clinical Blood Sciences and Cardiology, St George's University Hospitals NHS Foundation Trust and St George's University of London, UK

The authors have undertaken a prospective observational study to assess the additional value of the

measurement of ceramides for this prediction in patients with acute coronary syndrome (ACS) in a Chinese population. They demonstrate that addition of specific ceramides may improve diagnostic deficiency.
A significant problem with this manuscript is a lack of the relevant analytical information which is essential for a study such as the one described. The authors need to address the following points in the manuscript.

1. The authors use the term ACS in the title without declaring this as acute coronary syndrome.

Response：Many thanks for your suggestion. The term ACS has now been declared as acute coronary syndrome in the title.

2. Page 5 (introduction) second paragraph, line 3. Measurement of creatine kinase MB isoenzyme is no longer considered as a sensitive and specific marker of myocardial injury. Troponin alone is used. This statement should therefore be modified.

Response: Thank you and we totally agree with your comment. The statement has now been modified to “Among biomarkers, cardiac troponins play a central role in establishing a diagnosis and stratifying risk. Troponins are more specific and sensitive than the traditional cardiac enzymes such as creatine kinase (CK), its isoenzyme MB (CK-MB), and myoglobin (Hamm, Christian W, 2011, Eur Heart J)”. (Section 1., Paragraph 2, Line 3-5)

3. Page 5 (introduction) line 3. “However, lesion low-density cholesterol etc”. What are the authors referring to in this statement. I presume they are referring to lipid content of atherosclerotic plaque. Please will they clarify this statement.

Response: We are sorry that the previous statement might cause confusion. We were referring to lipid content of atherosclerotic plaque. This statement has now been modified to “However, lesional LDL is known to be rich in ceramide in the atherosclerotic plaque, and it contains 10- to 50-fold-higher content of ceramide when compared with plasma LDL.” (Section 1., Paragraph 3, Line 4-6)

4. A cursory search of the literature reveals 4 other papers that examine ceramides and ACS. The authors mention only one of these in the discussion section. A statement that this is the first paper seems somewhat inappropriate. These references should also be incorporated both in the introduction and the discussion.
1. de Carvalho LP, Tan SH, Ow GS, Tang Z, Ching J, Kovalik JP et al. Plasma Ceramides as Prognostic Biomarkers and Their Arterial and Myocardial Tissue Correlates in Acute Myocardial Infarction. JACC Basic Transl Sci 2018; 3:163-175.
2. Anroedh S, Hilvo M, Akkerhuis KM, Kauhanen D, Koistinen K, Oemrawsingh R et al. Plasma concentrations of molecular lipid species predict long-term clinical outcome in coronary artery disease patients. J Lipid Res 2018; 59:1729-1737.
3. Laaksonen R, Ekroos K, Sysi-Aho M, Hilvo M, Vihervaara T, Kauhanen D et al. Plasma ceramides predict cardiovascular death in patients with stable coronary artery disease and acute coronary syndromes beyond LDL-cholesterol. Eur Heart J 2016; 37:1967-1976.

Response: We totally agree that the association between ceramides and ACS were examined in several previous studies. Our study is the first study focused on the demonstration of plasma ceramides levels in diagnosing ACS patients from patients with chest pain in a Chinese cohort. The Eur Heart J paper was discussed in the first version of manuscript: “A previous study applying ceramides to the prediction of cardiovascular death from patients with CAD showed that ceramides,

independent of other lipid markers and CRP, were significantly associated with CV death.[19] (Section 4, Paragraph 4, Line 5-7)"

The other two papers are now discussed in the manuscript: "Recent studies showed targeted profiling of ceramides predicted short-term and long-term major adverse cardiac events (MACE).[28 29] Moreover, a consistent increase in ceramide levels and overexpression of 3 enzymes in ceramide biosynthesis were found on rat ischemic myocardium, which is consistent with the elevated plasma levels of ceramides found in our cohort.[29] (Section 4, Paragraph 4, Line 11-15)"

5. Page 7. Laboratory methods. The authors need to supply the following information. What samples were taken for laboratory analyses (whole blood, serum or plasma), what sample tubes were used, the preparation and storage of samples prior to analysis. Duration of sample storage. In addition, for each of the analytes used in the study the authors need to supply significantly more information on the laboratory methodology. This can be supplied as a supplementary appendix but should include a brief overview of methodology (excluding abbreviations which will not be clear to the reader) detection limit, analytical range, imprecision profile and reference interval or decision limit as appropriate. In particular, the authors need to state for the cardiac Troponin T assays the 10% imprecision and 99th percentile reference interval used for diagnosis. In addition, if a male and female reference limits was used as is now recommended.

Response: Blood samples for ceramides test were collected and centrifuged using EDTA anticoagulation tube at admission. Plasma were immediately stored in -80℃ for future analysis. Ceramides test were taken immediately after samples of all patients were collected. (Section 2.4., Paragraph 2, Line1-3).

Troponin levels were measured by electrochemiluminescence method using high sensitive-cTnT assay (Roche Diagnostics) on Roche Cobas e601. The coefficient of variation in the hs-cTnT assay is ≤10% at the cut-off value of 0.013 ng/ml. The 99 percentile upper reference limit of hs-cTnT assay is 0.014 ng/ml. Besides, the assay also has a limit of blank of 0.003 ng/ml and a limit of detection of 0.005 ng/ml, and the analytical range is 0.003-10 ng/ml. Unfortunately, we don't use reference limits separately for male and female. The information is now reported in Section 2.2, Line 3-8. Detailed information of other analytes is summarized in Supplementary Table 1.

6. Page 7. Diagnosis of ACS. The diagnosis of myocardial infarction should be made using the international definition of myocardial infarction version 3, ideally version 4 in addition to American Heart Association (not abbreviated) guidelines. There is also a spelling error in the penultimate line "cardia" for cardiac.

Response: Many thanks for pointing out the issue. The diagnosis of myocardial infarction also included international definition of myocardial infarction version 3 in previous submitted manuscript. Section 2.3 was modified as following: "The diagnosis of ACS including UA, Non-ST segment elevation myocardial infarction (NSTEMI), and ST-segment elevation myocardial infarction (STEMI) was made by two independent cardiologists by reviewing all patients' notes, including symptoms, 12-lead ECG, and blood tests results according to the 2014 American Heart Association/American College of Cardiology guidelines and international definition of myocardial infarction version 3.[20 21]" (Section 2.3, Line 1-5)

Many thanks for pointing out the spelling error. It has now been corrected. (Section 2.3., Paragraph 1, Line 12).

7. Page 8. Ceramide methodology. Blood sampling needs to be specified (see above).

Were samples centrifuged prior to analysis? Instrumentation parameters should be included in the data appendix should the dilution protocol.

Response: Blood samples for ceramides test were collected and centrifuged using EDTA anticoagulation tube at admission. Plasma were immediately stored in -80℃ for future analysis (Section 2.4., Paragraph 2, Line 1-3). Instrumentation parameters and the dilution protocol are now reported in Section 2.4, Paragraph 1 & 2:

“The mass spectrometry was operated in multiple-reaction monitoring (MRM) mode with ESI-positive ionization. The capillary voltage was set at 3.0 kV. and the source temperature was 120℃. The desolvation temperature and gas flow were 400℃ and 800L/h, respectively. The source offset was maintained at 60V.”

“Before analysis, the samples were thawed at room temperature, then a volume of 800 μl of protein precipitation solution (isopropanol) that containing D7-Cer d18:1/16:0 (0.01 pmol/μl), D7-Cer d18:1/18:0 (0.005 pmol/μl), D7-Cer d18:1/24:0 (0.015 pmol/μl) and D7-Cer d18:1/24:1 (0.015 pmol/μl) was pipetted into 1.5 mL Eppendorff tube after addition of 50 μL of plasma sample.”

8. Page 13 and 14. The authors comment on the potential for improving diagnostic classification. However, I do not get a feeling for whether or not the measurement of ceramides is applicable in the routine clinical laboratory and the authors should make some comment on the feasibility. Certainly, I do not see this test being delivered in real time with a 60 minute turnaround.

Response: Many thanks for pointing out this issue. LC-MS technique has shown significant high sensitivity and accuracy in measuring lipid molecules and other small molecules. As for clinical laboratory practice, standardization of test protocol and automated pretreatment of samples will enable a quicker and more accurate delivery of test results in the near future.

In response to your concern and in order to fully cover the limitations of our study to the readers, we have rephrased the statement regarding the potential and limitation of ceramides measurement for improving diagnostic classification as following:

“An improvement in the test technique of ceramides, including standardization of test protocol and automated pretreatment of samples is also needed to fulfil the requirement of clinical practice.” (Section 4., Paragraph 2, Line 11-13)

Reviewer: 2
Reviewer Name: Arash Mokhtari

Institution and Country: Department of Emergency Medicine and Internal Medicine, and Department of Cardiology, Lund University, Skane University Hospital in Lund
Lund, Sweden

In this study by Yao et al. the authors aimed to evaluate the value of ceramides in the diagnosis of ACS in chest pain patients. The study population was chest pain patients, their index test was ceramide levels and their outcome a final diagnosis of ACS as adjudicated by 2 independent cardiologists. What the authors found was that (Cer(d18:1/24:1(15Z))/Cer(d18:1/24:0)) ratio, Cer(d18:1/14:0) and Cer(d18:1/22:0) were independent predictors of ACS. They then conclude that ceramides are of diagnostic value for detecting ACS.
This is a large multicenter study, and the premise is interesting. I do however have several concerns stated below:

Major:
1.The “study design and participants” section needs clarification.
1a: What this a planned analysis, or is this a secondary analysis of a study performed for another purpose?
1b: You state that you included chest pain patients who were admitted. Where they included after admission to a ward (and in that case, which wards?) or are you referring to ED admission? Who included patients, authors? Research nurses? During what time of day and which days of the week? Did you include patients 24/7 all days of the week?
1c: Why did you choose these specific exclusion criteria which are somewhat unusual for a chest pain study? You also state that patients with chronic kidney disease were excluded but what GFR did you use as cut-off for exclusion? You also have “other conditions” as an exclusion criteria, what exactly was included in this definition?
1d. Why did you include patients with STEMI? You state that your aim was to evaluate the value of ceramides for diagnosing ACS, but we do not rely on biomarkers for diagnosing patients with STEMI, which is a diagnosis based on the ECG. I would recommend excluding patients with STEMI as to be able to evaluate your biomarkers in the population you aim for it to be used clinically.
1e: I would like you to make a flowchart to include in the manuscript, and that you in the initial results section report how many who were screened for eligibility and how many who were excluded (reported for each exclusion criteria). This is part of the STROBE checklist (point 13), where you state that this is reported on p10, but it is not.
1f: Where there any missing data? Did all patients have blood samples stored? If not, what happened to patients who did not, where they excluded (not stated as exclusion criteria)?

Response: Many thanks for your insightful comments. Those details are critical for this study and we have now reported them in the manuscript:

1a: This is a planned analysis to evaluate the association between circulating lipid molecule levels and cardiovascular disease.

1b: Those patients were included in chest pain outpatient by authors of this paper after pre-screening by senior research nurses in the chest pain centers. Blood samples were collected at admission. The patients were included Monday to Friday of the week.

1c: Pregnant women and organ transplant patients are not eligible for coronary angiography. Patients suffering from bleeding disorders are contraindication of anticoagulant drug. Patients with chronic kidney disease may have abnormal troponin levels, which are not eligible to compare the diagnostic effects between troponin and ceramides. Other conditions include those were clearly diagnosed as non-cardiac chest pain in pre-screening.

Patients with chronic kidney disease and an eGFR＜60 mL/min/1.73m^2 were considered to suffer from contrast-induced nephropathy more easily during coronary angiography and were excluded from the study (Solomon R, 2006, Kidney Int Suppl.). (Section 2.1, Paragraph 1, Line 6).

1d: Thank you very much for your insightful comment. We totally agree that STEMI can be easily diagnosed by ECG. However, the goal of our study is to evaluate the diagnostic value and potential application of ceramides in detection of ACS at admission. Due to the primary study design, ACS including STEMI, NSTEMI, and UA were analyzed as a group.

As in clinical practice, we do not rely on biomarkers in diagnosing STEMI, levels of ceramides and others biomarkers in patients excluding STEMI are also analyzed and summarized in Supplementary Table 2 & Supplementary Table 3.

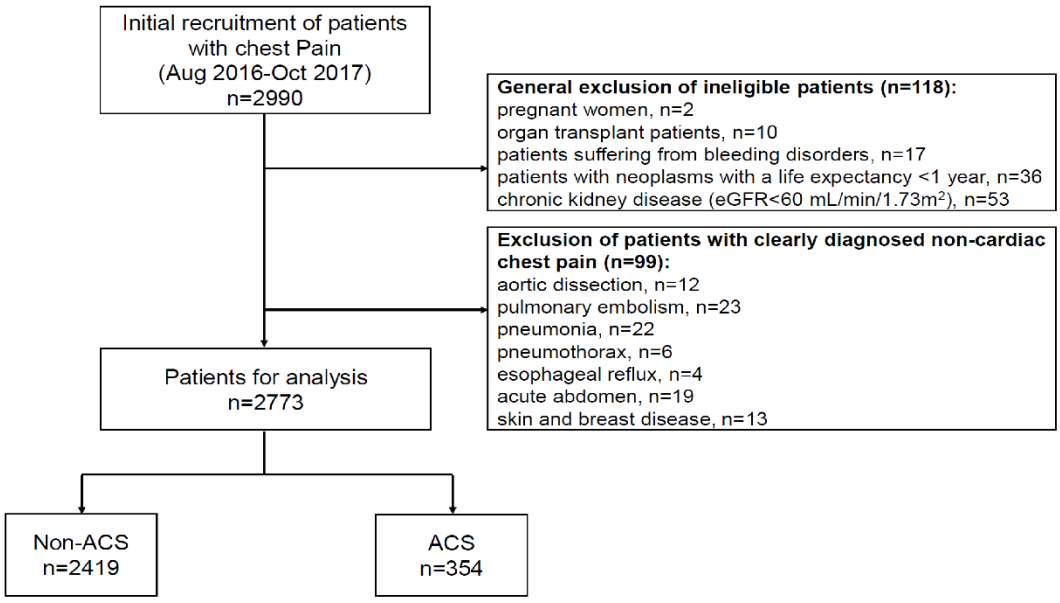
Results regarding hs-cTnT and ceramides levels in UA and non-ST-elevation acute coronary syndrome (NSTE-ACS) patients are summarized as following:

Parameters	Non-ACS (n=2419)	UA (n=116)	NSTE-ACS (n=230)
hs-cTnT (ng/mL)	0.008 (0.005-0.013)	0.008 (0.005-0.011)	0.049 (0.009-0.263) ###
Cer(d18:1/16:0)	0.198 (0.162-0.241)	0.242 (0.203-0.285)***	0.235 (0.201-0.286) ###
Cer(d18:1/18:0)	0.049 (0.036-0.065)	0.068 (0.052-0.080)***	0.066 (0.050-0.083) ###
Cer(d18:1/24:0)	2.053 (1.592-2.672)	2.218 (1.684-2.771)	2.183 (1.675-2.756)
Cer(d18:1/24:1(15Z))	0.569 (0.436-0.739)	0.764 (0.593-1.061)***	0.732 (0.552-0.950) ###
Cer(d18:1/14:0)	0.003 (0.002-0.004)	0.003 (0.003-0.004)***	0.003 (0.002-0.004) ###
Cer(d18:1/20:0)	0.051 (0.039-0.065)	0.063 (0.054-0.082)***	0.062 (0.052-0.078) ###
Cer(d18:1/22:0)	0.375 (0.294-0.480)	0.439 (0.350-0.594)***	0.413 (0.340-0.550) ###
Cer(d18:0/16:0)	0.010 (0.007-0.013)	0.013 (0.010-0.017)***	0.012 (0.009-0.016) ###
Cer(d18:0/18:0)	0.005 (0.003-0.007)	0.007 (0.005-0.011)***	0.007 (0.004-0.011) ###
Cer(d18:0/24:0)	0.060 (0.042-0.086)	0.075 (0.055-0.104)**	0.071 (0.052-0.096) ##
Cer(d18:0/24:1(15Z))	0.028 (0.019-0.041)	0.045 (0.032-0.065)***	0.042 (0.028-0.06) ###
Cer(d18:1/24:1)	0.258 (0.192-0.372)	0.333 (0.228-0.413)**	0.313 (0.229-0.389) ###
Cer(d18:1/16:0)/Cer(d18:1/24:0)	0.095 (0.079-0.114)	0.109 (0.090-0.133)***	0.107 (0.090-0.133) ###
Cer(d18:1/18:0)/Cer(d18:1/24:0)	0.023 (0.018-0.031)	0.031 (0.023-0.039)***	0.031 (0.023-0.039) ###
Cer(d18:1/24:1(15Z))/Cer(d18:1/24:0)	0.261 (0.211-0.345)	0.353 (0.267-0.481)***	0.327 (0.255-0.429) ###

UA vs. non-ACS: *p<0.05, **p<0.01, ***p<0.001. NSTE-ACS vs. non-ACS, #p<0.05 ##p<0.01, ###p<0.001. ACS, acute coronary syndrome; NSTE-ACS, non-ST-elevation acute coronary syndrome. UA, Unstable angina.

In patients diagnosed with whether UA or NSTE-ACS, levels of ceramides and ceramide ratios were also significantly higher compared to patients diagnosed with non-ACS (Supplementary Table 3), suggesting ceramides were also of diagnostic value in patients with UA and NSTE-ACS. In addition, this result suggests ceramides might be of diagnostic value of UA, which cannot be diagnosed with hs-cTnT.

1e: Many thanks for your suggestion. A flow chart describing the inclusion and exclusion process is now shown as Figure 1:



1f: All patients have blood samples stored and ceramides measurement were conducted immediately after recruitment of all patients was finished.

2. The “Diagnosis of ACS” section also needs further clarification.
2a: The diagnosis of ACS was based on adjudication by 2 independent cardiologists. But what did you do with the cases where the 2 cardiologists disagreed? Please also report the agreement level and kappa value between the adjudicators in the results section.
2b. Was ceramide levels available to the adjudicators or were they blinded to the index test?
2c. You specifiy that “Cardiac chest pain and elevations in troponins levels without ST elevation indicate NSTEMI.” The diagnosis of MI should however also be based on a significant rise/fall of troponin levels as well, as defined in the universal MI definition criteria (Thygesen et al. Fourth Universal Definition of Myocardial Infarction. 2018). Was serial troponin levels also part of the definition criteria your adjudicators used? If so, please report how a significant change in serial hs-cTnT was defined at different troponin time samplings.
2d: Did you include both type 1 and type 2 MI or only type 1?

Response:

2a: The cases where the 2 cardiologists disagreed were evaluated by a third cardiologist. The agreement level and kappa value between the adjudicators are now reported in Section 2.3, Paragraph 1, Line 5-7: “The agreement level and kappa value between the 2 cardiologists were 99.46% and 97.58%. The cases where the 2 cardiologists disagreed were reviewed by a senior cardiologist. All the cardiologists were blinded to ceramide levels.”

2b: All the cardiologists were blinded to ceramide levels and all lab technicians who were in charge for ceramides test were blinded to diagnosis.

2c: Serial troponin levels were measured at 0h-3h after admission in all recruited patients. Especially, in patients already diagnosed with myocardial infarction, troponin was also measured at 24h after admission. However, the diagnosis of myocardial infarction is based on a rise and/or fall of cTnT values with at least one value above the 99th percentile upper reference limit.

2d: We include both type 1 and type 2 myocardial infarction.

3. How was sample size determined? In the STROBE checklist point 10 you state that this is described in p7, but it is not.

Response: Many thanks for pointing out this issue. Now we have included the same size statement in the supplementary information: “Sample size: conventional sample size calculations based on the effect size and variation of data are generally not applicable for observational studies especially involving complex multivariate analyses. Instead, we use the concept of margin of error to give a general indication of the precision of our estimates. The margin of error for a particular statistic of interest is usually defined as the radius (or half the width) of the confidence interval for that statistic. A sample size of 2773 patients in our study will give a margin of error of 1.8%, at a 95% confidence level, which indicates a high level of accuracy in our results”.

4. You state that your aim was to “assess the value of ceramides in detection of ACS in patients with chest pain”. In clinical practice we are interested in knowing which patients have a low enough risk of having ACS and who can be discharged (ruled-out), and which patients who have a high enough risk and who should be admitted and for example undergo coronary angiography (ruled-in). With regards to rule-out, achieving this for MI is rather straight forward. We evaluate the ECG to exclude STEMI and we can thereafter use only the admission (0h) hs-cTnT in some lower risk patients and in most other chest pain patients we can use a 0h in combination with a second hs-cTnT after 1-3h and achieve a very high negative predictive value for MI. This is also the approach recommended by the European Society of Cardiology (Roffi et al. ESC guidelines NSTE-ACS 2015). If you want to show the added value of ceramides in this situation, you need to for example show that a combination of ceramides + hs-cTnT enables us to identify more patients for rule-out using only 0h testing (increased efficiency), or that adding ceramides to hs-cTnT testing yields an increase in sensitivity and negative predictive value (increased safety). I do not believe that you have shown this in this study. This approach would require calculating a cut-off to be used in clinical practice for the different ceramides. If you could do this, and present diagnostic accuracy measures (sensitivity, specificity, predictive values, likelihood ratios) for ceramides and hs-cTnT and when combined, this would better answer your aim of the study and provide data that I believe is more clinically applicable. This way I can as a reader easier draw conclusions regarding the potential clinical implications.
The primary difficulty of rule-out/rule-in is among patients with slightly elevated hs-cTnT and without a significant change on serial sampling. You state yourselves in the discussion that “However, high-sensitive troponin assays identify a larger number of patients with elevated troponin results but without a final diagnosis of ACS, making interpretation of test results challenging” which is a great point. But you have not shown that the addition of ceramides provides some added value in this context either, which also would require going about it as stated above for rule-out.
Finally, even though ruling out MI is rather straight forward in most cases, the diagnosis of UA is not. It would be interesting to see if ceramides could improve upon the diagnosis of UA, where we know that hs-cTnT by itself is clearly inadequate. This would again require calculating diagnostic accuracy measures and evaluating the potential added value.
Response: Many thanks for your insightful comments. We totally agree and understand your concerns on the clinical utility and application of our proposed method especially regarding rule-in, rule-out and cut-off values, which makes great sense in the traditional clinical setting especially with a single biomarker. However, in diagnosis or prognosis using multiple biomarkers and complex statistical models or machine learning techniques, the cutoff values are not generally recommended as all the biomarkers are assessed continuously and predicted outcomes are presented in probabilities due to the nature of multivariate statistical modelling. Also, the performances of such multivariate predictive models are commonly evaluated and summarized using the AUROC (area under the ROC curve) and NRI (net reclassification improvement). ROC curves are actually made of both sensitivity and specificity but give a better and more complete picture of the both. Examples of application of this kind can be found in the following papers:

1. Laaksonen R, Ekroos K, Sysi-Aho M, et al. Plasma ceramides predict cardiovascular death in patients with stable coronary artery disease and acute coronary syndromes beyond LDL-cholesterol. Eur Heart J 2016;37(25):1967-76. doi: 10.1093/eurheartj/ehw148

2. Bernal W, Wang Y, Maggs J, Willars C, Sizer E, Auzinger G et al. Development and validation of a dynamic outcome prediction model for paracetamol-induced acute liver failure: a cohort study. The Lancet Gastroenterology & Hepatology. 2016 Nov;1(3):217-225. https://doi.org/10.1016/S2468-1253(16)30007-3

However, the reviewer made an excellent point on rule-in and rule-out in clinical practices. This could be further investigated using the ROC curves of our models under the different assumptions of trade-offs between sensitivity and specificity. One formal and contemporary approach is the 'decision curve analysis':

- Vickers AJ, Elkin EB. Decision curve analysis: a novel method for evaluating prediction models. Med Decis Making. 2006; 26(6):565–74. [PubMed: 17099194]

Although good and relevant, it becomes very clinical and is beyond our primary focus on the association between ceramides and ACS. We realized that our study is just the start of a journey of using ceramides to detect ACS and more need to be done to make it clinically applicable therefore we added the following statement in the discussion: "Although our ceramides-based diagnostic model showed great potential in identifying ACS among patients with chest pain, its clinical utility especially regarding rule-in and rule-out strategies and performances still need to be further investigated and validated to make it fully applicable in clinical settings" (Section 4, Paragraph 2, Line 3-6).

Finally, regarding the diagnosis of UA, again it's a very good point. We found that in patients diagnosed with UA, levels of ceramides and ceramide ratios were significantly higher compared to patients diagnosed with non-ACS (Supplementary Table 3), suggesting ceramides were also of diagnostic value in patients with UA, which however could not be detected by hs-cTnT. We have now added the above findings in the main text (Section 3.2, Paragraph1, Line 8-10).

5. I may have misinterpreted this, but as I understand it Qlabs is a medical company that helped you with the analysis and interpretation of data. Several of the co-authors also seem to work for this company. Yet you state that there are no conflicts of interest. Does this company provide this analysis, and if so, I would believe that this is a clear conflict of interest.
Response: Many thanks for pointing out this issue. Now we have re-written the conflicts of interest statement to make it clear: "Q-life lab holds patents for the diagnostic use of ceramides for cardiovascular risk determination in China. WG, GX and CX work for Q-life and GX and CX are also shareholders. Other authors declare no conflict of interest." (Page 4)

Minor:
7. 12,8% of patients were diagnosed with ACS, how many were STEMI, NSTEMI and UA respectively?

Response: Among 354 patients who were diagnosed with ACS, 116 (4.2%) patients were UA, 114 (4.1%) patients were NSTEMI and 124 (4.5%) patients were diagnosed with STEMI.

8. How many of your patients' hade serial troponin measurements? At what time intervals did you measure serial hs-cTnT in these hospitals? Did all patients have hs-cTnT measurements?

Response: Yes. All patients have serial troponin measurements within 0-3h after admission. Especially, in patients already diagnosed with myocardial infarction, troponin was also measured at 24h after admission.

9. Among those defined as having UA, how many had objective findings such as ECG changes, pathological objective testing such as SPECT, CCTA, or a significant stenosis on coronary angiography?

Response: The diagnostic criteria for UA is reported in Section 2.3, Paragraph 1, Line 9-13: "Those with presence of 1 or more of 3 principal ischemic symptoms ((1) rest angina (lasting >20 minutes), (2) new-onset (<2 months previously) severe angina, and (3) a crescendo pattern of occurrence (increasing in intensity, duration, frequency, or any combination of these factors)) without elevations in cardiac troponins are defined as UA." The diagnosis of UA does not rely on imaging test including SPECT and CCTA, or a significant stenosis on coronary angiography. All included patients underwent ECG test and coronary angiography, but no SPECT or CCTA. Among patients diagnosed with UA (n=116), 26 patients had significant ST segment changes in ECG, and all patients had coronary stenosis of more than 50% in at least one coronary artery lumen area.

10. I would recommend re-phrasing the following in the discussion:
10a: "Ceramides measurement in high throughput quality-controlled environments is straightforward and cost-efficient". I do not believe you have shown in this study that measuring ceramide levels in chest pain patients is cost efficient.
10b. "By setting up clinical laboratories equipped with robotized sample handling systems and mass spectrometry equipment, it would be feasible and practical to identify patients with chest pain at high cardiovascular risk using our ceramides-based diagnostic model." I do not feel that you have shown why this would be practical (see also point 4 under "Major").
10c. "However, with our model, ACS risk evaluation can be improved with high precision, thereby avoiding delays in treatment for those at high risk and also avoiding invasive,
lengthy and expensive tests for those at low risk." I do not believe you have shown in this study that you could actually improve upon ACS diagnosis with ceramides (see point 4), nor that it would avoid delays or further testing.

Response: Thank you very much for your recommendations. We have rephrased those statement:

10a: "Ceramides measurement in high throughput quality-controlled environments is straightforward". (Section 4, Paragraph 3, Line 10-11)
10b: "By setting up clinical laboratories equipped with robotized sample handling systems and mass spectrometry equipment, it would be feasible to identify patients with chest pain at high cardiovascular risk using our ceramides-based diagnostic model." (Section 4, Paragraph 3, Line 11-14)

10c: A significant improvement in AUROC suggested an improvement of ACS diagnosis, including both sensitivity and specificity in diagnosing the disease. This might suggest avoiding invasive, lengthy and expensive tests for those suspected for ACS. We re-write the statement as "However, with our model, ACS risk evaluation can be improved with high precision, thereby avoiding invasive, lengthy and expensive tests for those suspected for ACS." (Section 4, Paragraph 5, Line 8-9).

11. In the limitations section please include a discussion on issues of generalizability, the fact that your results would need validation in other cohorts, as well as a discussion on other sources of potential bias such as selection bias.

Response: Many thanks for your suggestion. Limitation section has been revised to fully cover the issues of generalizability and selection bias: “The study was also possibly limited by a potential selection bias for more severe patients during recruitment process in chest pain outpatient of University affiliated hospitals. In addition, the results found in our study need to be validated in independent external cohorts.” (Section 4, Paragraph 2, Line 8-11)

12. I recommend some changes for the tables:
12a: There are too many decimals in several places
12b: Abbreviations should be explained below each table
12c: In table 1 please include more clinical data such as how many underwent further testing with echocardiography, coronary angiography or non-invasive testing (such as SPECT, CCTA, exercise ECG).
12d: The hs-cTnT levels presented in table 1 are ng/L and should therefore be corrected (i.e. not 0.009 but instead 9).
Response: Many thanks for your careful review and pointing out these issues. We have made all the changes according to your suggestions.

12a: All tables are checked and numbers with too many decimals have been simplified.

12b: Abbreviations are now explained below each table.

12c: All recruited patients underwent echocardiography, coronary angiography and ECG. No patient underwent APECT, CCTA or exercise ECG.

12d: We are sorry for the mistake and hs-cTnT levels are now presented as ng/ml.

13. I feel the STARD checklist would be more appropriate than the STROBE checklist for this study
Response: Many thanks for your suggestion, a STARD checklist is now shown in the manuscript.

VERSION 2 – REVIEW

REVIEWER	Professor Paul CVollinson St George's University Hospitals NHS Foundation Trust UK
REVIEW RETURNED	08-Mar-2019

GENERAL COMMENTS	The authors have addressed the majority of the issues raised in the previous review. There are some minor additional changes that need to be made however to complete the answer the points raised. 1. The characteristics of the troponin assay should be reported according to the recommendations of the International Federation of Clinical Chemists in nanograms/litre and whole numbers (limit of blank 3 ng/L limit of detection 5 ng/L etc). In this regard in the supplementary table the authors suggest the decision limit for cardiac troponin is 30 ng/L. The current recommended 99th percentile is 14 ng/L which is the requirement for diagnosis using the universal definition of myocardial infarction. Please will the authors clarify and state categorically what the decision limit used to classify patients in the study was. 2. The authors have added some methodology but unfortunately are using abbreviations (COD-PA and GPO-PAP). Unfortunately these are meaningless and need to be spelt out fully.

	3. In the supplementary table the authors do not include the assay imprecision range of the analytes measured in the study.

REVIEWER	Arash Mokhtari Department of Emergency Medicine and Internal Medicine, and Department of Cardiology, Lund University, Skane University Hospital in Lund, Sweden
REVIEW RETURNED	01-Mar-2019

GENERAL COMMENTS	I appreciate that the authors have provided an adequate response to most of my concerns. I only have some further minor comments: 1. With regards to my previous question (1b) I'm still not clear on the answer. Where patients recruited from the emergency department or did you only include patients who were admitted to a ward/chest pain unit? In the methods section you state that you included patients who were admitted. In the limitations section you have however added that they were outpatients. But you also state that all patients underwent coronary angiography, were they then not admitted? If you only included admitted patients, then your patients have a somewhat higher risk of ACS as compared to an unselected emergency department chest pain cohort which also includes lower risk patients who are discharged from the emergency department. This makes the results less generalizable to emergency department chest pain patients and should therefore be addressed in the limitations section. You should also clarify in methods section 2.1 from which wards patients were recruited ie if it was from chest pain units, CCU etc. If they were recruited from chest pain units, that means that patients at the highest risk of ACS who were admitted to the CCU also were not included? Where did you recruit the STEMI patients? 2. In methods section 2.1 you state that 2806 patients were recruited. But your flowchart shows that 2990 patients were recruited. Please correct this. 3. You responded to several of my concerns, but have not included the following in the manuscript itself: 3a. You replied that you included patients during Monday to Friday. This should be mentioned under "study design and participants". 3b. You replied that all patients had a 0h and 3h hs-cTnT measured and all patients underwent echocardiography and coronary angiography. Please add this information under section 3.1 of Results. You can then remove the following sentence which has been added beneath table 1: "All recruited patients underwent echocardiography, coronary angiography and ECG. No patient underwent APECT, CCTA or exercise ECG." 3c. You replied that you included both type 1 and type 2 MI in your outcome, please add this under section 2.3 "diagnosis of ACS" 3d. You replied that of 354 patients with ACS, 116 (4.2%) were UA, 114 (4.1%) NSTEMI, and 124 (4.5%) were STEMI. Please add this under section 3.1 of Results. 3e. You replied that of 116 patients with UA, all had a significant stenosis on coronary angiography. Please add this information under section 3.1 of Results.

	3f. You replied that you also excluded patients with a clear non-cardiac chest pain. Please add this information to your other exclusion criteria listed in methods section 2.1 4. The kappa value should be reported as 0.98 instead of as a percentage. 5. I would still like the following sentence from the discussion removed or rephrased: “However, with our model, ACS risk evaluation can be improved with high precision, thereby avoiding invasive, lengthy and expensive tests for those suspected for ACS.” I don’t believe that you have shown that by using ceramides we could avoid further testing, neither serial troponins, nor non-invasive testing or coronary angiography. I can’t use the current results and apply them to my patients to avoid further testing as that would require actual cut-offs to be used clinically and diagnostic accuracy parameters of that cut-off showing added value to using only hs-cTnT. I understand that you want this paragraph to discuss potential future clinical implications. But you could then in this paragraph instead briefly mention that ceramides levels were higher in patients with UA, as compared to patients without ACS. Ruling out MI is rather straight forward in most cases using hs-cTnT, while the diagnosis of UA is not. If Ceramides could provide an objective way of identifying patients with UA, this would have clinical implications. You could also mention that as ceramide levels where higher in NSTEMI patients compared to non-ACS, there could perhaps be a role for ceramides in distinguishing between patients with elevated hs-cTnT due to ACS vs non-ACS conditions. This should however only be seen as hypothesis generating and would need further studying to see if this really is the case. 6. The study design is described as “cross-sectional epidemiological survey” in the abstract. Prospective observational study is perhaps a better description.

VERSION 2 – AUTHOR RESPONSE

Reviewer: 2 Reviewer Name: Arash Mokhtari

Institution and Country: Department of Emergency Medicine and Internal Medicine, and Department of Cardiology, Lund University, Skane University Hospital in Lund, Sweden

I appreciate that the authors have provided an adequate response to most of my concerns. I only have some further minor comments:

1. With regards to my previous question (1b) I’m still not clear on the answer. Where patients recruited from the emergency department or did you only include patients who were admitted to a ward/chest pain unit? In the methods section you state that you included patients who were admitted. In the limitations section you have however added that they were outpatients. But you also state that all patients underwent coronary angiography, were they then not admitted? If you only included admitted patients, then your patients have a somewhat higher risk of ACS as compared to an unselected emergency department chest pain cohort which also includes lower risk patients who are discharged from the emergency department. This makes the results less generalizable to emergency department chest pain patients and should therefore be addressed in the limitations section. You

should also clarify in methods section 2.1 from which wards patients were recruited ie if it was from chest pain units, CCU etc. If they were recruited from chest pain units, that means that patients at the highest risk of ACS who were admitted to the CCU also were not included? Where did you recruit the STEMI patients?

Response: We are sorry that we did not response clearly to your last comment(1b). There might be some misunderstanding arising from differences in medical process and practices between hospitals in European countries and in China. In our study, 2990 patients included were all admitted to wards of cardiology in hospitals rather than emergency department. The statement in the Methods Section has been revised as “A total of 2990 patients with chest pain were consecutively recruited in chest pain outpatient during Monday to Friday between August 2016 and October 2017, and 2773 patients were finally admitted to wards of cardiology after pre-screening by the exclusion criteria.” (Section 2.1, Line 2-4)

However, these admitted patients with cardiac chest pain all firstly underwent the pre-screening in the chest pain outpatient. In our hospital most patients with chest pain usually firstly arrive at chest pain outpatient, and after primary examination and evaluation by the cardiologists these patients are admitted to wards of cardiology if they have suspected cardiovascular diseases, among whom clearly diagnosed diseases of non-cardiac chest pain including aortic dissection, pulmonary embolism and acute abdomen were excluded. Therefore, we admit that patients in our study have a higher risk of ACS compared to those in emergency department. Discussion regarding this issue has been rephrased as “The study was possibly also limited by a potential selection bias for patients with higher risk of ACS during recruitment process from admitted patients rather than patients in emergency departments.” (Section 4, Paragraph 2, Line 8-10).

Furthermore, it is necessary to clarify that patients in our study are admitted to wards of cardiology, which include both general wards of cardiovascular diseases and CCU for patients with fatal risks. In addition, we do not have the setting of “chest pain units” in our hospital. Most of STEMI patients are firstly sent to emergency department due to acute and severe symptoms, but other STEMI patients with atypical or slight symptoms may firstly come to the chest pain outpatient. Thus, we recruited STEMI patients in the chest pain outpatient.

2. In methods section 2.1 you state that 2806 patients were recruited. But your flowchart shows that 2990 patients were recruited. Please correct this.

Response: Many thanks for pointing out the mistake. It has now been corrected as “A total of 2990 patients with chest pain were consecutively recruited in chest pain outpatient during Monday to Friday between August 2016 and October 2017, and 2773 patients were finally admitted to wards of cardiology after pre-screening by the exclusion criteria.” in Section 2.1., Line 2-4.

3. You responded to several of my concerns, but have not included the following in the manuscript itself:

3a. You replied that you included patients during Monday to Friday. This should be mentioned under “study design and participants”.

3b. You replied that all patients had a 0h and 3h hs-cTnT measured and all patients underwent echocardiography and coronary angiography. Please add this information under section 3.1 of Results. You can then remove the following sentence which has been added beneath table 1: “All recruited patients underwent echocardiography, coronary angiography and ECG. No patient underwent APECT, CCTA or exercise ECG.”

3c. You replied that you included both type 1 and type 2 MI in your outcome, please add this under section 2.3 “diagnosis of ACS”

3d. You replied that of 354 patients with ACS, 116 (4.2%) were UA, 114 (4.1%) NSTEMI, and 124 (4.5%) were STEMI. Please add this under section 3.1 of Results.

3e. You replied that of 116 patients with UA, all had a significant stenosis on coronary angiography. Please add this information under section 3.1 of Results.

3f. You replied that you also excluded patients with a clear non-cardiac chest pain. Please add this information to your other exclusion criteria listed in methods section 2.1

Response: Apologies for this. We totally agree that these responses should be included in manuscript. They are now included accordingly:

3a: We now report that “This is a prospective observational study involving four University affiliated hospitals in Shanghai, China. A total of 2990 patients with chest pain were consecutively recruited in chest pain outpatient during Monday to Friday between August 2016 and October 2017, and 2773 patients were finally admitted to wards of cardiology after pre-screening by the exclusion criteria.” in Section 2.1., Line 2-4.

3b. We now report that “Serial troponin levels were measured at 0h-3h after admission in all recruited patients and all patients underwent echocardiography and coronary angiography.” in Section 3.1, Paragraph 1, Line 1-3. The statement beneath table 1: “All recruited patients underwent echocardiography, coronary angiography and ECG. No patient underwent APECT, CCTA or exercise ECG.” has been deleted.

3c. In Section 2.3, Line 5, we added that “Type 1 and type 2 MI were both included.”

3d. “In 2773 patients with chest pain, 354 (12.8%) were diagnosed with ACS, among whom 116 (4.2%) were UA, 114 (4.1%) were NSTEMI, and 124 (4.5%) were STEMI.” is now reported in Section 3.1, Paragraph 1, Line 3-5.

3e. “All 116 patients with UA had a significant stenosis on coronary angiography.” is now reported in Section 3.1, Paragraph 1, Line 5.

3f. “The exclusion criteria were pregnant women; organ transplant patients; patients suffering from bleeding disorders; patients with neoplasms with a life expectancy <1 year; chronic kidney disease (eGFR＜60 mL/min/1.73m2) and patients with a clear non-cardiac chest pain.” is now reported in Section 2.1., Paragraph 1, Line 6-9.

4. The kappa value should be reported as 0.98 instead of as a percentage.

Response: Many thanks for pointing out this issue and it has been corrected. “The agreement level and kappa value between the 2 cardiologists were 0.99 and 0.98.” (Section 2.3, Line 6)

5. I would still like the following sentence from the discussion removed or rephrased: “However, with our model, ACS risk evaluation can be improved with high precision, thereby avoiding invasive, lengthy and expensive tests for those suspected for ACS.” I don’t believe that you have shown that by using ceramides we could avoid further testing, neither serial troponins, nor non-invasive testing or coronary angiography. I can’t use the current results and apply them to my patients to avoid further testing as that would require actual cut-offs to be used clinically and diagnostic accuracy parameters of that cut-off showing added value to using only hs-cTnT.

I understand that you want this paragraph to discuss potential future clinical implications. But you could then in this paragraph instead briefly mention that ceramides levels were higher in patients with UA, as compared to patients without ACS. Ruling out MI is rather straight forward in most cases using hs-cTnT, while the diagnosis of UA is not. If Ceramides could provide an objective way of identifying

patients with UA, this would have clinical implications. You could also mention that as ceramide levels where higher in NSTEMI patients compared to non-ACS, there could perhaps be a role for ceramides in distinguishing between patients with elevated hs-cTnT due to ACS vs non-ACS conditions. This should however only be seen as hypothesis generating and would need further studying to see if this really is the case.

Response: We are thankful to your careful evaluation and suggestions about the statements in our manuscript. We totally agree with your comment and the original sentence has been removed. Additionally, the following statements discussing the potential clinical value of ceramides in identifying patients with UA and NSTEMI have been added:

“Noteworthily, ceramides levels of patients with UA are higher than those without ACS, which indicates that ceramides may help in identifying patients with UA and higher risk of ACS from populations without significant elevation of cardiac troponins. Moreover, ceramides levels were higher in NSTEMI patients compared to non-ACS ones, indicating a possible role of ceramides in distinguishing between patients with elevated troponins caused by ACS or non-ACS conditions. Nevertheless, the potential roles of ceramides in clinical diagnosis of UA and NSTEMI need further independent investigation and validation.” (Section 4, Paragraph 5, Line 8-14)

6. The study design is described as “cross-sectional epidemiological survey” in the abstract. Prospective observational study is perhaps a better description.

Response: Many thanks for your constructive comment. The study design has been described as “Prospective observational study” throughout the manuscript.

Reviewer: 1

Reviewer Name: Professor Paul CVollinson

Institution and Country: St George's University Hospitals NHS Foundation Trust UK

The authors have addressed the majority of the issues raised in the previous review. There are some minor additional changes that need to be made however to complete the answer the points raised.

1. The characteristics of the troponin assay should be reported according to the recommendations of the International Federation of Clinical Chemists in nanograms/litre and whole numbers (limit of blank 3 ng/L limit of detection 5 ng/L etc).

In this regard in the supplementary table the authors suggest the decision limit for cardiac troponin is 30 ng/L. The current recommended 99th percentile is 14 ng/L which is the requirement for diagnosis using the universal definition of myocardial infarction. Please will the authors clarify and state categorically what the decision limit used to classify patients in the study was.

Response: Many thanks for detailed evaluation of our manuscript and pointing out these issues. The characteristics of the troponin assay are now reported as ng/L across the manuscript and supplementary information.

In our study, the decision limit for highly sensitive cardiac troponin is 30ng/L, which we admit is different from 14ng/L, the 99th percentile in recommended guidelines for diagnosing myocardial infarction. However, the higher decision limit applied in our hospitals are due to avoiding over-diagnosis of myocardial infarction, especially in elderly patients. Furthermore, our decision limit is also supported by statements and recommendations of laboratorians in Norway, and here we quote

"Meanwhile, laboratorians in Norway have recommended not using the 99th percentile as the cutoff for MI, but rather a higher level of 0.03ug/L." from an article entitled 'Here Come High-Sensitivity cTn Assays Why Labs Need to Gear up Now' by Genna Rollins in AACC Clinical Laboratory News, February 2011, volume 37, number 2.

2. The authors have added some methodology but unfortunately are using abbreviations (COD-PA and GPO-PAP). Unfortunately these are meaningless and need to be spelt out fully.

Response: We are sorry that these abbreviations were not spelt out and might be confusing. They are now described as "total cholesterol (TC) was measured by enzymatic cholesterol method using cholesterol oxidase/peroxidase aminophenazone (COD-PAP) reagent while total triglyceride (TG) was measured by Glycerol-3-Phosphate oxidase/peroxidase anti-peroxidase method (GPO-PAP) method." (Section 2.2, Line 8-11)

3. In the supplementary table the authors do not include the assay imprecision range of the analytes measured in the study.

Response: Many thanks for pointing out this issue. The assay imprecision range are now reported in Supplementary Table 1.

VERSION 3 - REVIEW

REVIEWER	Prof Paul Collinson St George's University Hospitals, UK
REVIEW RETURNED	01-May-2019

GENERAL COMMENTS	Minor comments only Supplementary table 1 (and elsewhere as appropriate) Apo E in mg/L, NTproBNP in ng/L

REVIEWER	Arash Mokhtari Department of Emergency Medicine and Internal Medicine, and Department of Cardiology, Lund University, Skane University Hospital in Lund, Sweden
REVIEW RETURNED	03-May-2019

GENERAL COMMENTS	I'm satisfied with the response by the authors and feel they have improved upon their initial manuscipt considerably. I have no further comments.

VERSION 3 – AUTHOR RESPONSE

Reviewer: 1

Reviewer Name: Prof Paul Collinson

Institution and Country: St George's University Hospitals, UK

Please state any competing interests or state 'None declared': None declared

Please leave your comments for the authors below

Minor comments only

Supplementary table 1 (and elsewhere as appropriate) Apo E in mg/L, NTproBNP in ng/L

Response: We appreciate that you gave positive evaluation to our manuscript and provide us with an opportunity to make minor revisions in our paper. The unit of measurement for ApoE in supplementary tables and Table1 has been changed correspondingly to mg/L, and the unit of NTproBNP has been changed to ng/L, respectively.

Reviewer: 2

Reviewer Name: Arash Mokhtari

Institution and Country: Department of Emergency Medicine and Internal Medicine, and Department of Cardiology, Lund University, Skane University Hospital in Lund, Sweden

Please state any competing interests or state ‘None declared’: None declared

Please leave your comments for the authors below

I'm satisfied with the response by the authors and feel they have improved upon their initial manuscipt considerably. I have no further comments.

Response: We are thankful to your comment and approval of our manuscript.