PEER REVIEW HISTORY

ARTICLE DETAILS

TITLE (PROVISIONAL)	Duivenvoorden, Annet; Clarysse, Mathias; Ceulemans, Laurens; Geelkerken, Robert H.; Derikx, Joep; de Vries, Jean-Paul; Buscher, Hessel; Damink, S.; van Schooten, Frederik; Lubbers, Tim; Lenaerts, K.; DMIS, Dutch Mesenteric Ischemia Study group
AUTHORS	Duivenvoorden, Annet; Clarysse, Mathias; Ceulemans, Laurens; Geelkerken, Robert H.; Derikx, Joep; de Vries, Jean-Paul; Buscher, Hessel; Damink, S.; van Schooten, Frederik; Lubbers, Tim; Lenaerts, K.; DMIS, Dutch Mesenteric Ischemia Study group

VERSION 1 – REVIEW

REVIEWER	Kazmi, Syed Sajid Hussain Oslo University Hospital, Department of Vascular Surgery
REVIEW RETURNED	05-Mar-2023

GENERAL COMMENTS	Study protocol submitted for publication with BMJ open. BMJ open invited the review with the following instructions and aims: 1- to ensure that the protocol is scientifically credible, 2- it is presented in an appropriate context, 3- the design is ethically and procedurally sound, and 4- thoroughness of review. As per instructions by the BMJ Open editorial office, it is not required a judgement on the priority or breadth of appeal of the submitted study protocol. Competing interests: I have nothing to declare and no competing interests. Documents available for review on the BMJ Open account 1- Note from the editor: Instructions for reviewers of study protocols 2- Study protocol 3- PDF Textbox and Figure1 4- STARD 5- SPIRIT guidelines Is there any major flaw in the study that would prevent a sound interpretation of the data? No. Comment 1: Within the submitted study protocol, corresponding author is mentioned as Kaatje Lenaerts, but in the email from BMJ Open editorial office, 22nd February, Miss Duivenvoorden is referred as contact author.

	Abstract: Aim of the study is to validates panel of plasma biomarkers and investigate the potential of volatile organic compounds (VOCs) analysis in exhaled air. Methods and analysis: Multicentre prospective observational study. Inclusion criteria and study population: 120 patients >18years of age with a clinical suspicion of AMI. Comment 2: Under point 4) the physician's consideration to perform a computed tomography scan, needs clarification. Does it mean that based on clinical manifestation, findings on physical examination and laboratory tests when it is suspected that the patients might be suffering from AMI, the physician will consider CT? And at this point the patient gives consent, and it will be followed by Patient's characteristics, Repetitive blood samples and Exhaled air will be collected. The authors will examine in the collected blood samples 1-plasma I-FABP 2-Villin-1 (VIL-1) 3-smooth muscle 22 (SM22). All these three biomarkers of intestinal ischemia will be assessed with ELISA. The exhaled air collected will be tested with gas chromatography time-of-flight mass spectrometry of VOCs to identify an AMI-specific profile. However, the diagnosis of AMI shall be based on CT findings, endovascular and surgical reports, and clinical findings. In addition (if applicable) verified by histopathological examination to determine the ischemic changes in the specimen. Approvals and registry: TACTIC trial protocol is approved by (METC azM/UM), the Netherlands (METC 19-010) and Ethics Committee Research UZ/KU Leuven, Belgium (S63500) and also local committees of the Dutch participating centres. ClinicalTrials.gov NCT05194527 Study recruitment start: July 2020 and is still ongoing. Primary outcomes: mentioned in the ClinicalTrials.gov : plasma I-FABP, VIL-1, and SM22 in the study patients. The sensitivity and specificity will be determined compared to the current gold standard, i.e., CTA. Secondary outcomes: Identification of specific VOC profiles in the exhaled air of the study patients. Identification of novel pathophysiologic pathways involved in AMI. Comment 3: Insufficient information and could be given more information if the authors can any specific VOC for AMI? The protocol version under review has been the version 4 (February 24, 2020) Issue date: 20-03-2020. The authors have mentioned a protocol amendment in terms of a change of primary sponsor. The authors mentioned that the protocol manuscript has been written according to the SPIRIT reporting and STARD guidelines and have also given references.

	Comment 4: In the protocol manuscript they have categorically mentioned the strength and limitations of TACTIC trial. Although a number of mesenteric ischemia biomarkers in the patients with AMI have been evaluated in previous studies it is first time that in TACTIC trial that a panel of ischemia biomarkers will be tested prospectively in a multicenter international cohort. To the best of the reviewer's knowledge it is first time that the data on the breath analysis in the patients with AMI. Introduction Background The authors have given a relevant description of AMI, epidemiology, seriousness, and the current clinical practice for its investigation. They have also mentioned appropriate references. The authors correctly mentioned CTA as the current gold standard modality for the diagnosis of AMI with an estimated sensitivity and specificity of 89-100%. However, the authors claim that it is an over estimation since the CTA studies (given references) had study cohort primarily consisted of patients with advanced mesenteric ischemia and did not include early or progressive mesenteric ischemia. Besides the authors also claim (with references) that a considerable %age of patients with AMI presents without ischemia-specific CT signs overlapping with other acute abdominal complications. They presume that there is a need for a more accurate, readily available, minimally invasive AMI specific test which can be performed quickly in the patients with even early phases of the suspected AMI. Comment 5: However, SM22 with 15 min half-life and in their previous study detected only 4 hours after the initiation of intestinal ischemia might not fulfill this early detection aim. Besides lack of detectable plasma levels of SM22 if used alone may result in a false negative interpretation of a patients with mucosal intestinal ischemia. In the study used for sample size calculation (reference 27) I-FABP seems to be a better biomarker of AMI since it was detectable during the earlier mucosal as well as during the late transmural ischemic phase. Further they discuss I-FABP as shown in earlier studies on rats and human intestinal ischemic models as a potential marker for earlier mucosal damage, and VIL-1 as a maker of a persisting ischemic mucosal damage due to its more extended period of detectability in plasma as compared to I-FABP. According to the authors the current intestinal ischemia biomarkers do not provide insight regarding the possible development of transmural ischemia and these known markers (I-FABP and VIL-1) represent only the mucosal ischemic damage (Comment 6: Please see the comment 5). They refer to plasma SM22 as a biomarker which can differentiate between mucosal and transmural intestinal ischemia injury. However, there is limited knowledge of these ischemia biomarkers specificity for AMI.

	Further in the background the authors discuss the potential of VOCs in the exhaled air in the patients with AMI. Identification of such a marker specific for AMI is attractive being a non-invasive technique. However, the challenge is to find one such marker since there are identified numerous VOCs. Another challenge in finding such a disease specific marker is that the VOCs composition is influenced by exogenous and endogenous factors. The authors presume that the VOC profile identified previously for IBD is expected to aid in AMI diagnosis since the two diseases share the pathophysiological processes. Comment 7: I think this part of the study will be more challenging to identify such one disease specific VOC for AMI. It would have been informative if the authors could add more information about the methods for analysis of exhaled air samples based on the recommendations of the ISO-11843 guidelines. See Filipiak W, et al. A compendium of volatile organic compounds (VOCs) released by human cell lines. Curr Med Chem. 2016; (23):2112-2131. However, it is positive that through TACTIC trial which is a prospective and an international multicentre design will come closer to identify a long awaited biomarker for AMI. The authors aim to include 60 patients with AMI and 60 or more patients with another clinical condition as a control arm. Comment 8: AMI is an emergency and the screening for inclusion of the patients with clinically suspected AMI will occur at one of the participating centres may inherit delay in the treatment. Informed written consent may not be possible in the patients in bad general condition and will further prolong inclusion/ study time for a disease with already low incidence. Procedure: Serial blood and exhaled air samples before and after endovascular and surgical treatment of AMI patients and also of the non-AMI participants upto 5 days. Besides histopathology of the specimens. Arterial blood samples via an arterial line, intravenous or central venous catheter. Vacutainer tubes EDTA for plasma and SST II advance tubes for serum specimens. Centrifuged and plasma/serum to storage tubes. Storage at -80 degrees C. Analysis with ELISA kits for I-FABP and SM22 developed at their own lab and ELISA kit for VIL-1 at PharmAbs Belgium. Exhaled air in resistance-free plastic bags (Tedler bag, UK), further stabilized on carbon desorption tubes and stored at 4 degrees C. Gas chromatography time of flight mass spectrometry will be performed. Study hypotheses: With the use of serum/plasma biomarkers I-FABP, VIL-1 and SM22, a timely diagnosis of patients with AMI before irreversible transmural bowel damage occurs can be achieved. Comment 9: The included patients (n=60) naturally will differ in the history of the initiation of the symptoms of AMI and will lead to subgroups with smaller sample size for a reliable data interpretation and any strong study conclusion. Comment 10: The authors have given insufficient details about VOCs and chemical methods planned to be utilized to discover novel pathophysiologic pathways involved in AMI.

	Sample Size Calculation: Comment 11: They have used the results of I-FABP from their previous study Schellekens, DHS et al. SM22 a plasma biomarker for human transmural intestinal ischemia. Ann Surg. 2018.268(1):120-126, for the sample size calculation. It would have been more clear for the readership, as well as the others planning a similar study to know the method used for the effect size determination, e.g., Cohen's d formula? Comment 12: Statistical analysis plan seems to be appropriate for the study data. They seem to have the appropriate ethical approvals and fundings for the conduction of this study. Overall recommendation: A group of clinicians and researchers with a long contribution in the topic of mesenteric ischemia. It is interesting and promising that they are now conducting another study to find a long awaited biomarker for AMI. In my humble opinion the current protocol for TACTIC study be accepted for publication.

REVIEWER	Kurt, Nezahat Yildirim University, MEDICAL BIOCHEMISTRY
REVIEW RETURNED	04-Apr-2023

GENERAL COMMENTS	Excellent research and overall design. I would like to draw attention to a few points that need suggestions or explanations: - The inclusion and exclusion criteria for the non-AMI group from the study groups are not clear. Did this group have existing complaints from AMI patients? - What co-morbid diagnoses did the AMI patients have? - How many of the AMI patients had superior mesenteric artery embolism, mesenteric artery thrombosis, and non-occlusive mesenteric ischemia based on CT results? - Both serum and plasma samples were collected. Why not just serum or just plasma? Which analytes were studied in serum and which analytes in plasma? - Arterial blood is usually used for blood gas analysis, and venous blood is used for routine biochemistry. Why did you choose arterial blood in your study? - If a significant AUC is obtained on the ROC curve, it is recommended to calculate the positive and negative predictive values as well.

REVIEWER	Mege, D. Hospital Timonec
REVIEW RETURNED	09-Apr-2023

GENERAL COMMENTS	This is an interesting project. Biomarkers in acute mesenteric ischaemia are necessary to improve diagnosis and management. I have some comments: Methods: - The reason to include the same number of control patients as AMI patients is not clear. - The sample size is calculated with mean I-FABP levels, it's thus not correct to include in the primary objective the diagnostic

	accuracy of SM22 and VIL-1. These biomarkers should be analyzed in the secondary objective. - Inclusion criteria should be more detailed in the abstract, text and text box 1: which clinical manifestation? which physical finding? Which laboratory measurement? - The analysis between biomarkers and VOC and severity of AMI and prognosis could be considered in secondary objectives. Figure 1: - It would be more understandable to change T1, T2 etc by D1, D2 etc.. because it corresponds to day 1, day 2 etc and maybe for T0, T60 etc by H0, H60... - It's not clear if patients without intervention do not have T0, T60, T120 and T180 samples. - S in versus is missing. P14 L36 : Development without capital letter References 20, 21: issue number is missing

VERSION 1 – AUTHOR RESPONSE

Reviewer: 1

Comments to the Author:
Identifying an intestinal ischemia biomarker of sufficient sensitivity and specificity for routine clinical use has been challenging. Let alone identify biomarkers to differentiate between intestinal mucosal and transmural ischemia.
Congratulations on designing and getting the appropriate approvals for an international multicentre study for patients with AMI. Attached are some notes/ comments I made while reviewing this protocol.

We are grateful to the reviewer for the comments and recommendations to our protocol.

Comment 1: Within the submitted study protocol, corresponding author is mentioned as Kaatje Lenaerts, but in the email from BMJ Open editorial office, 22nd February, Miss Duivenvoorden is referred as contact author.

We have changed the corresponding author in the portal of BMJ open to Kaatje Lenaerts.

Comment 2: Under point 4) the physician's consideration to perform a computed tomography scan, needs clarification. Does it mean that based on clinical manifestation, findings on physical examination and laboratory tests when it is suspected that the patients might be suffering from AMI, the physician will consider CT?

We acknowledge the reviewer's comment and have rewritten the sentence in the section "Study Design and eligibility criteria" (Page 10-11) to clarify the inclusion criteria:

" Patient are eligible for study participation if they have a clinically suspicion of AMI, which is based on (1) the clinical manifestation of the disease, (2) physical examination by the local physician, (3) laboratory measurements and (4) the physician's consideration to perform a CT(A)-scan. If all criteria are met, the physician will contact the local research team and the patient or legal representative will be asked for informed consent to participate in the study. Clinical study procedures are initiated when all criteria are met and informed consent is obtained from the patient or legal representative. After inclusion, blood and exhaled breath will be collected at consecutive time points (Figure 1)."

Secondary outcomes: Identification of specific VOC profiles in the exhaled air of the study patients.

Identification of novel pathophysiologic pathways involved in AMI.

Comment 3: Insufficient information and could be given more information if the authors can any specific VOC for AMI?

It would indeed be advantageous to have more information on already identified VOC species in patients with AMI. Unfortunately, the analysis and identification of AMI specific VOC profiles has not been explored yet. The analysis of VOCs in exhaled air is a non-invasive technique that has already been demonstrated to differentiate between multiple clinical conditions, including inflammatory bowel disease and non-alcoholic steatohepatitis and healthy subjects. To our knowledge, no other studies are available that provide information regarding specific VOCs in patients with suspected AMI (with various etiologies), but some animal studies provide certain insights.

We included the following information into the introduction of this manuscript (Introduction, page 9):

"In 2011, a pilot study in the analysis of VOCs in rats following acute superior mesenteric artery (SMA) occlusion showed that they were able to identify a small cluster of VOCs that increase during ischemic bowel injury (Jimenez et al 2011). Other studies, have explored the possibility of monitoring exhaled methane (CH_4) concentrations in order to detect SMA malperfusion (Szilárd et al 2019). This is now being investigated in a prospective observational study in patients with trauma-related haemorrhage following blunt trauma (Jávor et al 2022). Based on these findings we could speculate that CH_4 concentrations could be relevant in our future measurements and analyses"

Still, at this moment we have no concrete evidence to support this.

Comment 4: In the protocol manuscript they have categorically mentioned the strength and limitations of TACTIC trial.
Although a number of mesenteric ischemia biomarkers in the patients with AMI have been evaluated in previous studies it is first time that in TACTIC trial that a panel of ischemia biomarkers will be tested prospectively in a multicenter international cohort.
To the best of the reviewer's knowledge it is first time that the data on the breath analysis in the patients with AMI.

We thank the reviewer for the adequate assessment. This supports our own observations and the need for conducting this study.

Background: The authors have given a relevant description of AMI, epidemiology, seriousness, and the current clinical practice for its investigation. They have also mentioned appropriate references. The authors correctly mentioned CTA as the current gold standard modality for the diagnosis of AMI with an estimated sensitivity and specificity of 89-100%. However, the authors claim that it is an over estimation since the CTA studies (given references) had study cohort primarily consisted of patients with advanced mesenteric ischemia and did not include early or progressive mesenteric ischemia. Besides the authors also claim (with references) that a considerable %age of patients with AMI presents without ischemia-specific CT signs overlapping with other acute abdominal complications.
They presume that there is a need for a more accurate, readily available, minimally invasive AMI specific test which can be performed quickly in the patients with even early phases of the suspected AMI.

Comment 5: However, SM22 with 15 min half-life and in their previous study detected only 4 hours after the initiation of intestinal ischemia might not fulfill this early detection aim. Besides lack of detectable plasma levels of SM22 if used alone may result in a false negative interpretation of a patients with mucosal intestinal ischemia. In the study used for sample size calculation (reference 27) I-FABP seems to be a better biomarker of AMI since it was detectable during the earlier mucosal as well as during the late transmural ischemic phase.

We are grateful to the reviewer for the comments and his view on the background of the disease and the study protocol. We would like to elaborate on our claim in the introduction regarding CT(A) imaging in patients with suspected AMI. We fully support that CT(A) imaging is currently the most accurate method for the diagnosis of AMI. Unfortunately, a considerable percentage of patients with AMI present without ischemia-specific CT signs, or CT signs showing overlap with other acute abdominal complications. We believe that there is a need for better tests to unambiguously detect AMI based on plasma/serum detectable biomarkers which will assist in determining the severity and progression of the disease at an early stage. Combined with currently available techniques such as CT(A) imaging, this would allow a more rapid diagnosis and clinical decision making in these patients and further improve patient outcome.

Indeed, the previous study revealed that SM22 half-life is around 14 minutes in rats and 23 minutes in humans (Ref 27: Schellekens et al 2018) which indicates that this biomarker reflects the actual ischemic stage.

We agree with the reviewer that SM22 will indeed only be detected if mucosal ischemia progresses to transmural ischemia, and hence does not indicate early damage with only mucosal involvement. However, when treatment is initiated within the first 12 hours after onset of symptoms, death rate is seriously reduced. Therefore, we believe that SM22 detection, in combination with IFAPB, can act as a warning signal to rapidly start intervention. Furthermore, our study population is expected to consist of patients with different stages of (sustained) ischemic mesenteric damage. Patients are included in this study based on a clinical suspicion for AMI, therefore we are unaware of the existence, etiology and stage of the AMI in these patients. This allows us to examine the functionality of this panel of biomarkers and VOC profiles in a realistic patient cohort. This study will provide a pragmatic view and straightforward evaluation of the usefulness of these 'future' diagnostic techniques. It would be desired in the future, to investigate these markers in the different subtypes of AMI, to fully determine their sensitivity and specificity.

Further they discuss I-FABP as shown in earlier studies on rats and human intestinal ischemic models as a potential marker for earlier mucosal damage, and VIL-1 as a maker of a persisting ischemic mucosal damage due to its more extended period of detectability in plasma as compared to I-FABP.

Comment 6: According to the authors the current intestinal ischemia biomarkers do not provide insight regarding the possible development of transmural ischemia and these known markers (I-FABP and VIL-1) represent only the mucosal ischemic damage. They refer to plasma SM22 as a biomarker which can differentiate between mucosal and transmural intestinal ischemia injury. However, there is limited knowledge of these ischemia biomarkers specificity for AMI.

Previous work of Schellekens et al (2018) has shown that I-FABP levels are elevated in patients with mucosal ischemic damage and transmural ischemic damage. However, I-FABP does not discriminate between these two groups. SM22 is significantly elevated in patients with transmural ischemia compared to patients with only mucosal ischemic damage. Hence, the combination of markers such as I-FABP, VIL-1 and SM22 would provide a better picture on the stage of the ischemic damage in the intestine. This finding however needs to be validated in larger patient cohorts as we attempt to accomplish with the current study.

Further in the background the authors discuss the potential of VOCs in the exhaled air in the patients with AMI. Identification of such a marker specific for AMI is attractive being a non-invasive technique. However, the challenge is to find one such marker since there are identified numerous VOCs. Another challenge in finding such a disease specific marker is that the VOCs composition is influenced by

exogenous and endogenous factors. The authors presume that the VOC profile identified previously for IBD is expected to aid in AMI diagnosis since the two diseases share the pathophysiological processes.

Comment 7: I think this part of the study will be more challenging to identify such one disease specific VOC for AMI. It would have been informative if the authors could add more information about the methods for analysis of exhaled air samples based on the recommendations of the ISO-11843 guidelines. See Filipiak W, et al. A compendium of volatile organic compounds (VOCs) released by human cell lines. Curr Med Chem. 2016; (23):2112-2131.

We agree with the reviewer that identification and application of VOCs as disease specific markers is still challenging. Translation to clinical practice as a diagnostic tool is mostly hampered due to confounding effects (such as exogenous and endogenous factors) resulting in a bias in obtained outcomes. Despite these limitations, it has already been proven that VOCs are able to differentiate between various pathologies in patients (Ref 32: Sethi et al 2013 and Ref 33: van Berkel et al 2008). Additionally, data obtained from the exhaled breath analysis will not only be used to identify AMI-specific biomarker, but also to investigate pathophysiological processes occurring in patients AMI. This will provide us with a better insight into expression of AMI-specific VOC profiles and opens up and new way of exploring VOCs a new diagnostic technique for future clinical studies.

We also added more information on the measurement and analysis of the obtained VOCs samples (Section: Exhaled Breath collection and analysis, page 13):

"The GC-tof-MS analysis was performed as described previously (Kienhorst, et al. 2023, reference 39)"

However, it is positive that through TACTIC trial which is a prospective and an international multicentre design will come closer to identify a long awaited biomarker for AMI.

We are grateful to the reviewer for the comments and detailed evaluation of the manuscript.

Comment 8: AMI is an emergency and the screening for inclusion of the patients with clinically suspected AMI will occur at one of the participating centres may inherit delay in the treatment. Informed written consent may not be possible in the patients in bad general condition and will further prolong inclusion/ study time for a disease with already low incidence.

Patient safety and treatment is always prioritized and is not influenced by the study (We like to refer to manuscript in the Section: Safety Consideration and withdrawal of participation, page 15). If a patient is suspected of AMI, the treating physician will contact the local (coordinating) researcher for written informed consent (ICF). Written ICF may be provided by the patient or, if the patient is unable to give consent, by a legal representative (if available). If the patient or legal representative is unable to give ICF, for example due to the patient's treatment or general condition, or if the patient does not understand the conditions for study participation, he/she will not be approached for inclusion. The downside of this approach is that patients with suspected AMI will be missed for inclusion. Therefore, it is possible that suitable patients cannot be included which could prolong the study period.

We added a new sentence to the manuscript to support the described statement (Section: Safety Considerations and withdrawal of participation, page 15):

"Patient safety and treatment is always prioritized and is not influenced by the study."

In the process of obtaining ethical approval, we first requested deferred consent for study participation. Unfortunately, we could not use deferred consent because it is an observational prospective study. In order to use deferred consent for a study, the patient must benefit from the clinical study procedures during the study (such as an intervention study) according to the CCMO, which is not applicable in our case. We did not refer to this process in the manuscript.

Comment 9: The included patients (n=60) naturally will differ in the history of the initiation of the symptoms of AMI and will lead to subgroups with smaller sample size for a reliable data interpretation and any strong study conclusion.

We agree with the reviewer that the patients with AMI will differ in their history of symptom onset which may require subgroup analysis. However, this is also how patients present in daily practice as well, making it a realistic study cohort. Detailed information on the patients' medical history, treatment, laboratory measurements, radiological imaging, etc. will be collected (Section: Study design and eligibility criteria, page 10) to allow reliable data interpretation and subgroup formation, if needed, and to avoid misclassification. Interestingly, the naturally occurring diversity in our cohort also allows to get a unique insight into different stages and subtypes of AMI and its progression over time.

Comment 10: The authors have given insufficient details about VOCs and chemical methods planned to be utilized to discover novel pathophysiologic pathways involved in AMI.

We agree with the comments of the Reviewer and added information on the chemical methods for the discovery of novel pathophysiologic pathways involved in AMI (Also, see comment 7):

Section: Exhaled breath collection and analysis (page 13)

"Collected exhaled air containing VOCs is stabilized on carbon desorption tubes (SU60520-60-S, Camsco) with a Flow air sampling pump (LFS-113, 360-041-01, Sensidyne) and stored at 4°C until further analysis by gas chromatography time of flight mass spectrometry (GC-tof-MS) (37). The GC-tof-MS analysis was performed as described previously (Kienhorst, et al. 2023 or reference 39)"

Sample Size Calculation:

Comment 11: They have used the results of I-FABP from their previous study Schellekens, DHS et al. SM22 a plasma biomarker for human transmural intestinal ischemia. Ann Surg. 2018.268(1):120-126, for the sample size calculation. It would have been more clear for the readership, as well as the others

planning a similar study to know the method used for the effect size determination, e.g., Cohen's d formula?

We used indeed the Cohen's d formula to determine the effect size. We added this information to the manuscript (section Sample size, page 15).

Statistical analysis plan seems to be appropriate for the study data. They seem to have the appropriate ethical approvals and fundings for the conduction of this study.

Overall recommendation:

A group of clinicians and researchers with a long contribution in the topic of mesenteric ischemia. It is interesting and promising that they are now conducting another study to find a long awaited biomarker for AMI. In my humble opinion the current protocol for TACTIC study be accepted for publication.

We are grateful to the reviewer for the comments and kind words about this study protocol and our study team.

Reviewer: 2

Excellent research and overall design. I would like to draw attention to a few points that need suggestions or explanations:

Comment 1: The inclusion and exclusion criteria for the non-AMI group from the study groups are not clear. Did this group have existing complaints from AMI patients?

Patients are eligible to participate in the study upon initial suspicion of AMI. After completion of the diagnostic workup, including CT(A) imaging, the initially suspected AMI is eventually confirmed in a part of the patients (AMI group). The rest will have another diagnosis (non-AMI group). Diagnosis of AMI will be based on CT, endovascular and surgical reports, clinical findings, and (if applicable) verified by histopathological examination.

Hence, all eligible patients show clinical signs that can be found in AMI, and the inclusion and exclusion criteria for both AMI and non-AMI groups are the same (see, inclusion and exclusion criteria main text manuscript). We expect that AMI is the definitive diagnosis in about half of the included patients.

We changed the text in the "Study Design and Eligibility Criteria" to clarify the inclusion and exclusion criteria (page 10):

"Patients clinically suspected of AMI will be screened for inclusion at one of the participating centres. All study participants must fulfil the study inclusion criteria and will be excluded from participation if they cannot provide written informed consent or do not fulfil the inclusion criteria (Text Box 1: Inclusion and

exclusion criteria). Patient are eligible for study participation if they have a clinically suspicion of AMI, which is based on (1) the clinical manifestation of the disease, (2) physical examination by the local physician, (3) laboratory measurements and (4) the physician's consideration to perform a CT(A)-scan. If all criteria are met, the physician will contact the local research team and the patient or legal representative will be asked for informed consent to participate in the study. Clinical study procedures are initiated when all criteria are met and informed consent is obtained from the patient or legal representative. After inclusion, blood and exhaled breath will be collected at consecutive time points (Figure 1)"

Comment 2: What co-morbid diagnoses did the AMI patients have?

The study is still ongoing and we did not perform any analysis on this subject. Based on literature, we can predict that a vast majority of patient with AMI in our study will present with several comorbidities and risk factors. There are a limited number of studies that give a detailed description of common comorbidities in patients with AMI (Marchena-Gomez et al 2009, Chen et al 2020, Witte et al 2022). In these studies Charlson comorbidity index (CCI) was used to determine the frequency of certain comorbidities in patient with (suspected) AMI, and most importantly predict the short-term outcome, morbidity, mortality and Quality of Life (QoL) in long term survivors. The study from Marchena-Gomez (2009) investigated possible comorbidities in 186 patients who underwent surgery for AMI (Table below).

List of comorbidities according to Charlson Comorbidity Index score (Marchena-Gomez et al 2009)

Condition	Score	No. of patients (%)
Myocardial infarction	1	33 (17.7)
Congestive heart failure	1	20 (10.8)
Peripheral vascular disease (lower limb ischemia)	1	70 (37.6)
Cerebrovascular disease	1	39 (21.0)
Dementia	1	14 (7.6)
Chronic obstructive pulmonary disease	1	41 (22.0)
Connective tissue disease	1	4 (2.2)
Peptic ulcer disease	1	18 (9.7)
Mild liver disease	1	2 (1.1)
Diabetes	1	19 (10.2)
Hemiplegia	2	2 (1.1)
Moderate-severe renal disease	2	21 (11.3)
Diabetes with organ damage	2	32 (17.2)
Any tumor (within past 5 years)	2	20 (10.8)
Lymphoma	2	0 (0.0)
Leukemia	2	0 (0.0)
Moderate-severe liver disease	3	15 (8.1)
Metastatic solid tumor	6	5 (2.7)
AIDS	6	0 (0.0)

The incidence of AMI increases exponentially with age, therefore it is known that AMI is more prevalent in patient above 75 and older. In 2021, the new guidelines for AMI were presented during the World

Society of Emergency Surgery Congress. In 2022, this statement was published and the guidelines were updated (Bala et al 2022). In this publication, they also mentioned an overview of common risk factors found in patient with different types of AMI (Table below).

Table 1: Risk factors for specific types of AMI (Acute mesenteric ischemia: updated guidelines of the World Society of Emergency Surgery, Bala et al 2022)

	Pathogenesis of AMI			
	Acute mesenteric arterial embolism	**Acute mesenteric arterial thrombosis**	**NOMI**	**Mesenteric venous thrombosis**
Risk factors	Atrial fibrillation recent MI cardiac thrombi Mitral valve disease Left ventricular aneurysm Endocarditis Previous embolic disease	Diffuse atherosclerotic disease Postprandial pain Weight loss	Cardiac failure Low flow states Multiorgan dysfunction Vasopressors Abdominal compartment syndrome	Portal hypertension history of VTE Oral Contraceptives Estrogen use Thrombophilia Pancreatitis
Clinical onset	Sudden strong abdominal pain, vomiting	Progressive or sudden abdominal pain, vomiting, diarrhea and/or melena	Progressive pain, mild	Nonspecific GI symptoms, abdominal distension, worsening of general condition
Vascular involvement	Main artery or branches of SMA	Celiac trunk, SMA, IMA origins	Superior mesenteric vein, progression to portal vein	Stenosis of SMA

Comment 3: How many of the AMI patients had superior mesenteric artery embolism, mesenteric artery thrombosis, and non-occlusive mesenteric ischemia based on CT results?

We please like to refer to our previous answer on comment 2. The TACTIC study is still ongoing and we did not yet investigate the occurrence of SMA embolism, mesenteric artery thrombosis and NOMI. Based on literature we can make an estimate on the number of cases with these specific subtypes (Bala et al 2022). Around half of cases of AMI are caused by an acute SMA embolism and thrombosis of the SMA occurs in around 25% of all cases. NOMI is found in approximately 20% of cases, whereas patients with mesenteric venous thrombosis only account for less than 10% of all cases of AMI (Bala et al 2022).

Comment 4: Both serum and plasma samples were collected. Why not just serum or just plasma? Which analytes were studied in serum and which analytes in plasma?

In our panel, I-FABP and SM22 are measured in the collected plasma samples, whereas VIL-1 is detected in serum. We also now mentioned the following on the the measurement of VIL-1 in serum (Section: Blood Collection and biomarker analysis, page 12-13):

"VIL-1 is detectable in plasma, however earlier studies observed a better detection in serum compared to plasma (Ceulemans et al in preparation)". Therefore, evaluation of VIL-1 will be done in serum samples.

Comment 5: Arterial blood is usually used for blood gas analysis, and venous blood is used for routine biochemistry. Why did you choose arterial blood in your study?

To reduce patient discomfort, blood samples will be collected via an existing arterial or, central venous catheter, or an intravenous needle if present. If it remains difficult to withdraw blood through these means a separate venapuncture can be performed to collect blood samples. Earlier studies on the currently investigated biomarkers revealed that plasma proteins concentrations were almost identical in either arterial or venous blood (unpublished). A study by Kelly et al (2013), showed that there is not difference in biomarker concentration between arterial and venous plasma samples in patients with chronic obstructive pulmonary disease. In addition, we have observed that je majority of our samples are currently collected either by intravenous needle or a separate venapuncture.

Comment 6: If a significant AUC is obtained on the ROC curve, it is recommended to calculate the positive and negative predictive values as well.

We agree with the reviewer and we added to the following to our statistical approach (Section: Statistical analysis):

"The area under curve (AUC) the receiver operating characteristics (ROC) curve (AUC-ROC) will be calculated and used to determine diagnostic utilities (sensitivity, specificity, positive predictive value and negative predictive value) of the biomarkers I-FABP, SM22, and VIL-1 to discriminate between AMI or non-AMI patients. Statistical analysis of VOCs expression profiles will be performed according to the published standards by Horvath et al. for the exhaled breath analysis….."

Reviewer: 3

Comments to the Author:

This is an interesting project. Biomarkers in acute mesenteric ischaemia are necessary to improve diagnosis and management.

I have some comments:

Comment 1: Methods:

The reason to include the same number of control patients as AMI patients is not clear.

When designing the study, we expected that for every two patients with AMI suspicion, one patient will have a confirmed AMI diagnosis. This might be an overestimate, and therefore, we are allowed to include more non-AMI patients (control) as we mentioned in the protocol the following "We may include a higher percentage of patients without mesenteric ischemia due to its non-specific clinical presentation and low overall incidence. Therefore, study inclusions will be finalized when 60 patients with confirmed AMI are included." Thus, we might end up with a larger non-AMI group, resulting in an unequal sample size per group.

Comment 2: The sample size is calculated with mean I-FABP levels, it's thus not correct to include in the primary objective the diagnostic accuracy of SM22 and VIL-1. These biomarkers should be analyzed in the secondary objective.

The effect size was determined based on differences in mean I-FABP levels between AMI and non-AMI patients. With this effect size, in combination with power 0.80 and alpha 0.05, we would need 41 patients per group. Since we have three outcome parameters, we will correct for multiple testing with Bonferroni. Therefore, we calculated the sample size with alpha 0.05/3, which leads to sample size of 54. Therefore, all three biomarkers will be analyzed as primary outcome. We clarified this in the manuscript text (section Sample size, page 15).

Comment 3: Inclusion criteria should be more detailed in the abstract, text and text box 1: which clinical manifestation? which physical finding? Which laboratory measurement?

We are grateful to the reviewer for the comments and we adjusted the inclusion and exclusion criteria to the following:

Changes in the abstract (Section: Abstract, page 4):

"Methods and analysis: In this international multicentre prospective observational study, 120 patients (> 18 years of age) will be recruited with clinical suspicion of AMI. Clinical suspicion of AMI is based on: (1) clinical manifestation, (2) physical examination, (3) laboratory measurements, and (4) the physician's consideration to perform a computed tomography (CT) scan. The patient's characteristics, repetitive blood samples, and exhaled air will be prospectively collected. Plasma levels of mucosal damage markers intestinal fatty acid binding protein and villin-1, as well as transmural damage marker smooth muscle 22, will be assessed by enzyme-linked immunosorbent assay. Analysis of VOCs in exhaled air will be performed by gas chromatography time-of-flight mass spectrometry. Diagnosis of AMI will be based on CT, endovascular and surgical reports, clinical findings, and (if applicable) verified by histopathological examination."

Changes in the main text: (Section: Study design and eligibility criteria, page 10-11 and Text Box 1):

"All study participants must fulfil the study inclusion criteria and will be excluded from participation if they cannot provide written informed consent or do not fulfil the inclusion criteria (Text Box 1: Inclusion and exclusion criteria). Patients are eligible for study participation if they have a clinically suspicion of AMI, which is based on (1) the clinical manifestation of the disease, (2) physical examination by the local physician, (3) laboratory measurements and (4) the physician's consideration to perform a CT(A)-scan. If all criteria are met, the physician will contact the local research team and the patient or legal representative will be asked for informed consent to participate in the study…."

We also made adjustments to Text box 1 by adding the following:

- *Clinical manifestation of the disease: sudden abdominal pain, nausea/vomiting, abdominal distention, diarrhea, haematochezia, haematemesis, tenderness and signs of peritonitis*
- *Physical examination and vital signs such as body temperature, heart rate, blood pressure, etc.*
- *Laboratory measurements such as white blood cell count, GST, D-lactate*
- *Physician's consideration to perform a CT(A)-scan*

Comment 4: The analysis between biomarkers and VOC and severity of AMI and prognosis could be considered in secondary objectives.

We agree it would be interesting to further explore. However, with our current sample size and limited exhaled breath samples, we are not able to look for differences between biomarkers and VOCs on the severity of AMI and prognosis. This would require more patient samples and is out of the scope of this study. However, based on the findings in this study, we will explore the relation between the biomarkers/VOCs/severity and prognosis.

Comment 5: Figure 1:

It would be more understandable to change T1, T2 etc by D1, D2 etc.. because it corresponds to day 1, day 2 etc and maybe for T0, T60 etc by H0, H60...

We agree with the reviewers and changed the labels for the follow up timepoints from T1, T2 by D1 and D2. However, we did not alter the other timepoints, because they refer to a timepoint on a similar day.

Comment 6: It's not clear if patients without intervention do not have T0, T60, T120 and T180 samples.

We also obtain the T0, T60, T120 and T180 samples from patients without an intervention. In most cases, within three hours a decision is made by the physician what the treatment strategy is for the patient. Therefore, it is possible that not all samples (T0 – T180) are obtained in the case of an earlier intervention (such as vascular or surgical intervention).

Comment 7: S in versus is missing.

We have added the s in versus.

Comment 8: P14 L36: Development without capital letter

We change the word 'Development' to one without a capital letter.

Comment 9: References 20, 21: issue number is missing

We added the issue number to the described references.

Because, we made some additional changes to the manuscript, more references were added. Therefore, the references number for these studies have changed to 29 and 31.

VERSION 2 – REVIEW

REVIEWER	Kurt, Nezahat Yildirim University, MEDICAL BIOCHEMISTRY
REVIEW RETURNED	29-May-2023

GENERAL COMMENTS	It is suitable for publication.