PEER REVIEW HISTORY

ARTICLE DETAILS

TITLE (PROVISIONAL)	Glutathione infusion before primary percutaneous coronary intervention: a randomized controlled pilot study.
AUTHORS	Tanzilli, Gaetano; Truscelli, Giovanni; Arrivi, Alessio; Carnevale, Roberto; Placanica, Attilio; Viceconte, Nicola; Raparelli, Valeria; Mele, Rita; Cammisotto, Vittoria; Nocella, Cristina; Barillà, Francesco; Lucisano, Luigi; Pennacchi, Mauro; Granatelli, Antonino; Dominici, Marcello; Basili, Stefania; Gaudio, Carlo; Mangieri, Enrico

VERSION 1 - REVIEW

REVIEWER	Ahmed N. Mahmoud Department of Cardiovascular Medicine, University of Florida, USA
REVIEW RETURNED	20-Aug-2018

GENERAL COMMENTS	In this multicenter randomized placebo controlled trial, the authors assess the impact of glutathione infusion on myocardial perfusion and cell survival indexes. The idea is rather novel and aside for small animal model studies, there is not enough evidence available in the current literature. The manuscript is well written and easy to understand. However, I do have some concerns regarding the current manuscript that I will highlight below: Major concerns: -Although results of the current study are interesting and thought provoking, the small sample size and the evaluation of non-clinical surrogates of reperfusion and myocardial survival is a large drawback of the current study. Thus, I would encourage the authors to down play the tone of the current manuscript and avoid strong statements (e.g. the abstract conclusion). I would recommend adding a sentence at the end of the conclusion section stating that future larger trial adequately powered for evaluation of clinical endpoints are needed to confirm the current finding. The impact of a certain intervention on non-clinical surrogates (although sometimes unavoidable due to small sample size) does not necessarily mean a similar impact on important clinical outcomes such as cardiovascular mortality or heart failure hospitalizations. A recent example is the use of aspiration thrombectomy in primary PCI. Although prior single centered small trials showed benefit in both non-clinical surrogates and even clinical outcomes, larger adequately powered trials failed to confirm such benefit.

	Minor concerns: Abstract: -Spelling and grammatical mistakes including misspelling the word “Abstract” itself. -In the results, the authors report progressive decrease of cTpT levels however stating a positive correlation coefficient r=0.41 going to figure 2B, it became clear that the r value reflected the changes in H2O2 in relation with the changes in cTpT. Recommend rephrasing the results to reflect this. -Recommend adding PCI to the keywords. Introduction: -The authors leaped to conclusions without citing evidence to back the claim when they stated the following “Over the time, this may result in adverse left ventricular (LV) remodeling and worse LV function.”. Can you please cite the evidence behind this statement? Methods: -Can the authors explain the limited enrollment period (March-August 2017)? Such a limited period resulted in a small population size, one of the major limitations of the study. Was there any reason for not including a larger sample size that could have had enough power to assess clinical outcomes? -The authors use hypothesis as endpoints both in primary and secondary endpoints. For example: Primary Endpoint should be (Biochemical markers of cell death) rather than (drug administration up to 3 days after procedure would attenuate ROS induced myocardial damage as assessed by measuring biochemical markers of cell death). Same goes with the secondary endpoints. -Primary Endpoint/s, biochemical markers of cell death, should be clearly stated in the methods section. -How were the secondary myocardial perfusion endpoints assessed (cTFC and TMPG)? who assessed those outcomes? Why didn’t the authors consider a more objective way of assessment e.g. MRI or Myocardial perfusion scintigraphy? - what were the adverse events evaluated? Did it include worsening renal function? Results: -No major comments on reporting the study results. Discussion: -The reviewer anticipated that the authors are going to explain the reason why clinical endpoints were not utilized as well as the 5-day limit the authors used for the echocardiography imaging without a follow up period. The authors need to clarify the reason behind the small sample size and limiting the inclusion period to 6 months. Conclusions: -The conclusions were appropriate, and the authors concluded mainly based on the results of the study. (recommend rephrasing the abstract conclusion to something similar) Tables: -Appropriate with the information clearly detailed. Figures:

	-Line numbers ran over parts of the figures making them hard to read

REVIEWER	Fournier S Aalst, Belgium
REVIEW RETURNED	20-Aug-2018

GENERAL COMMENTS	In this trial, authors evaluate whether the infusion of GSH before acute reoxygenation in STEMI patients might counteract the deleterious effects of increased H2O2 generation on myocardium. The study is very interesting, the design also, but to a formal point of view, several issues are present and should be solved. For example, regarding troponins: in the on-line protocol, this is one of the secondary endpoint, in the manuscript, this is the primary endpoint, in the method section, they describe the use and calculations of AUC, and in the results, they don't provide them. Effort should be made to make everything consistent. Major issues that should be solved : 1. There is a major issue regarding the endpoint of the trials (A. Their description and B. differences with the online protocol). A. The endpoints should me described more precisely. In the method section, the description of the primary/secondary endpoints looks like hypotheses. Regarding primary outcome, they state "biochemical markers of cell death" and I assume that they refer to troponins. They should describe in details (which test ? AUC or not ? Which time points ? …). The abstract and the results section should focus on this primary outcome first, and then on the secondary endpoints. For the secondary endpoint, they mention cTFC and TMPG. It should be more precise. In table 2, they report MPG ≥2 and MPG=3. Similarly, they report mean cTFC and cTFC<20. What were the exact secondary outcomes ? B. Very surprisingly, on EudraCT website (and not "EuROdraCT"), the primary and secondary endpoints are different. According to the original protocol, the primary endpoint consisted in the assessment of the effects of the infusion of "glutatione sale sodico" on the reduction of the oxidative markers and inflammation after PCI and the secondary endpoints were 1) the assessment of the variations of the corrected TIMI frame count (cTFC) and the TIMI Myocardial Perfusion Grade (TMPG) after PCI; 2) the assessment of the middle values of peak of the cardiac Troponin, after the procedure; 3) To verify,through telephone contact or a programmed visit, the principal adverse clinical events as death, acute myocardial infarct, stent's thrombosis of the treated vessels or the occurence of a new revascularization, up to 6 months after the procedure. 2. The number of patients scheduled was 90, and they included 50. What about the sample size calculation ? What about this reduction to 50 ? 3. In the limitation section, authors write that their study is imitated by the lack of clinical end points and the small sample size. According to the original protocol, MACE were supposed to be an

	endpoint and the sample size was supposed to be twice higher. Could they comment? 4. Authors describe that the AUC for troponin release was measured for each patient. Where are these values ? Troponins are supposed to be the primary endpoint surrogate, and they are not presented according to the method AUC). 5. They introduce Table 1 and Table 2 by writing “Clinical and angiographic characteristics of patients are shown in Table 1 and 2. The baseline characteristics were well balanced between the two groups”. However, it appears that table 2 does not contain only baseline characteristics but also endpoints (cTFC, blush). 6. Authors report a « tight correlation between percentage changes of H2O2 and cTpT levels from baseline to 5 days was found in treated group (Figure 2B) ». However, the r coefficient is less than 0.5 and accordingly, this relationship is only modest. Furthermore, they should perform a multivariate analysis taking into account the many other factors able to influence troponin release (vessel, delays, etc). I would also invert the X and Y axes. 7. Apparently, an external Core Lab processed the data. Which corelab ? 8. Safety data (GSH) should also been included. 9. Authors should explain the rational of the protocol chosen for GSH administration (dosis of GSH, intervals,..) based on the literature / previous work.

REVIEWER	Shahar Lavi London Health Sciences Centre, Western University, Canada
REVIEW RETURNED	21-Aug-2018

GENERAL COMMENTS	This is a novel small pilot study evaluating the potential protective effect of gluthatione in patients with STEMI. The authors should be congratulated for performing the study. Overall the manuscript is well written. Make sure to follow consort criteria and rules; flowchart, etc. The endpoints are written as hypothesis and not as endpoints. When referring to H2O2 levels, how do you know that its production? And if measured in peripheral blood, that it affected the heart? Check accuracy of numbers. For instance: Table 1- killip class percentage incorrect. How do you define ischemia time. Did all patients have occluded artery initially? Please provide initial TIMI flow and if there a difference between patients with occluded artery at baseline or not.

	Please assess difference between LAD and other IRA. Troponin- the description in text is not clear. The figure is more meaningful. But my understading that it was measured every 6h. Please add those time points. There was no difference in echo results. Can glutathione affect troponin lab measurement without true effect on myocardial injury? Myocardial function- There is no difference. Please don't call it a trend in the results section or discussion. Discussion- first paragraph too strong for the results.

REVIEWER	Guido Knapp Department of Statistics TU Dortmund University Germany
REVIEW RETURNED	20-Oct-2018

GENERAL COMMENTS	Comments on statistics: Section on Statistical Analysis - Replace "normal distribution of parameters" by "normal distribution of continuous variables" Results section - Population, Table 1, Killip class, GSH group: It is 2 (8) not 2 (80). - Table 3: For baseline data, the p-value is from a two-sample test. How were the p-value calculated for Reperfusion and Follow-up? Are really the mean values and standard deviations of interest? I would expect mean changes here that is calculating the difference from Reperfusion to Baseline for each patient and the computing mean and standard deviation and finally compare the mean changes of the two groups. I hope that the authors addressed the problem of possible unequal variances in the groups properly in the applied test. (This sentence is not only related to Table 3 but to all tests with continuous variables.) - Table 4, Follow-up, Placebo: Typo LVEF SD 3.2 or 3.0 but not 3.2.0 -Table 4: I would first present the GSH group results than the placebo like in Tables 1 to 3. What is the meaning of * at P-value?

VERSION 1 – AUTHOR RESPONSE

Reviewer: 1

Although results of the current study are interesting and thought provoking, the small sample size and the evaluation of non-clinical surrogates of reperfusion and myocardial survival is a large drawback of the current study. Thus, I would encourage the authors to down play the tone of the current manuscript and avoid strong statements (e.g. the abstract conclusion). I would recommend adding a sentence at the end of the conclusion section stating that future larger trial adequately powered for evaluation of clinical endpoints are needed to confirm the current finding. The impact of a certain intervention on non-clinical surrogates (although sometimes unavoidable due to small sample size) does not necessarily mean a similar impact on important clinical outcomes such as cardiovascular mortality or heart failure hospitalizations. A recent example is the use of aspiration thrombectomy in primary PCI. Although prior single centered small trials showed benefit in both non-clinical surrogates and even clinical outcomes, larger adequately powered trials failed to confirm such benefit.	We thank the reviewer for the helpful suggestions. Accordingly, we changed abstract and conclusions. (see Abstract, page 2, Lines 21-23 and Discussion section, page 14, lines 11-13).
Abstract: -Spelling and grammatical mistakes including misspelling the word “Abstract” itself.	We rechecked all the manuscript for typos and misspelling
Abstract: -In the results, the authors report progressive decrease of cTpT levels however stating a positive correlation coefficient r=0.41 going to figure 2B, it became clear that the r value reflected the changes in H2O2 in relation with the changes in cTpT. Recommend rephrasing the results to reflect this	As suggested, we modified the results in the abstract (see page 2 line 19).
Abstract: -Recommend adding PCI to the keywords.	As suggested, we added PCI as key word (see page 2 line 26).
Introduction: -The authors leaped to conclusions without citing evidence to back the claim when they stated the following “Over the time, this may result in adverse left ventricular (LV) remodeling and worse LV function.”. Can you please cite the evidence behind this statement?	We have added some references addressing the role of coronary microcirculatory impairment on post-reperfusion LV recovery of function (see page 4 line 10 and references 8-10).

Methods: -Can the authors explain the limited enrollment period (March-August 2017)? Such a limited period resulted in a small population size, one of the major limitations of the study. Was there any reason for not including a larger sample size that could have had enough power to assess clinical outcomes? -The authors use hypothesis as endpoints both in primary and secondary endpoints. For example: Primary Endpoint should be (Biochemical markers of cell death) rather than (drug administration up to 3 days after procedure would attenuate ROS induced myocardial damage as assessed by measuring biochemical markers of cell death). Same goes with the secondary endpoints. -Primary Endpoint/s, biochemical markers of cell death, should be clearly stated in the methods section. -How were the secondary myocardial perfusion endpoints assessed (cTFC and TMPG)? who assessed those outcomes? Why didn't the authors consider a more objective way of assessment e.g. MRI or Myocardial perfusion scintigraphy? - what were the adverse events evaluated? Did it include worsening renal function? Results: -No major comments on reporting the study results.	We thank the reviewer for these comments. Accordingly, we rephrased the methods section to clarify the reviewer's concerns (see page 6, lines 4-6). We also provided the sample size calculation to justify our study population (see page 9, lines 13-19). Moreover, we choose a short time period of sample storage to avoid deterioration of the markers of interest in the different centers (see page 8, lines 8-12).
Discussion: -The reviewer anticipated that the authors are going to explain the reason why clinical endpoints were not utilized as well as the 5-day limit the authors used for the echocardiography imaging without a follow up period. The authors need to clarify the reason behind the small sample size and limiting the inclusion period to 6 months.	The paper is a pilot study and is aimed to explore whether intravenous GSH administration, just before acute reoxygenation of infarct myocardial areas and after wide recanalization, is able to blunt oxidative status thus reducing myocardial cell damage (see page 5, lines 1-10). It is conceivable that 5-days follow-up is too-short time to demonstrate LV recovery of function inside areas where stunned and hibernated myocardium coexist.
Conclusions: -The conclusions were appropriate, and the authors concluded mainly based on the results of the study (recommend rephrasing the abstract conclusion to something similar)	As suggested, we modified the abstract's conclusions. (see page 2, lines 21-23)
Tables: -Appropriate with the information clearly detailed.	We amended tables.

Figures: -Line numbers ran over parts of the figures making them hard to read	We amended Figures.

Reviewer: 2

1 A. The endpoints should me described more precisely. In the method section, the description of the primary/secondary endpoints looks like hypotheses. Regarding primary outcome, they state “biochemical markers of cell death” and I assume that they refer to troponins. They should describe in details (which test ? AUC or not ? Which time points ? …). The abstract and the results section should focus on this primary outcome first, and then on the secondary endpoints. or the secondary endpoint, they mention cTFC and TMPG. It should be more precise. In table 2, they report MPG ≥2 and MPG=3. Similarly, they report mean cTFC and cTFC<20. What were the exact secondary outcomes ?	We thank the reviewer for these comments. In the revised version of the methods section we clarified endpoints, gave more details on laboratory assessment and we modified the test for wording consistency. As suggested, the description of primary and secondary end-points has been rewritten (see page 7, lines 21-26 and page 8 lines 1-2). On the other hand, in the coronary microvasculature, post ischemic reperfusion results in endothelial cell damage, mainly driven by heightened oxidative activity, that contribute to the no-reflow phenomenon. Both, cTFC and MBG, are well recognized indices of impaired tissue perfusion at microcirculatory level and predict adverse left ventricular remodelling and mortality after primary PCI. In particular, TIMI flow ≤2 is associated with an increased risk of adverse remodelling at 6 months (J Am Coll Cardiol 2004;43:534–541) and of 5-year mortality (J Am Coll Cardiol 2010;55:2383–2389). Myocardial blush grade 0–1 increases the risk of adverse remodelling at 6 months (J Cardiol 2006;98:725–728) and of total mortality after 16 months of follow-up (Circulation 2003;107:2115–2119). Our preliminary data show that blunted oxidant activity correlates with the degree of improvement of reperfusion indices. Despite that, in order to make the message of the paper more homogeneous we have decided to remove such data by focusing the results on reduction of oxidative stress markers.
1 B. Very surprisingly, on EudraCT website (and not “EuROdraCT”), the primary and secondary endpoints are different. According to the original protocol, the	We apologize for the discrepancy and we clarified this issue.

primary endpoint consisted in the assessment of the effects of the infusion of “glutatione sale sodico” on the reduction of the oxidative markers and inflammation after PCI and the secondary endpoints were 1) the assessment of the variations of the corrected TIMI frame count (cTFC) and the TIMI Myocardial Perfusion Grade (TMPG) after PCI; 2) the assessment of the middle values of peak of the cardiac Troponin, after the procedure; 3) To verify,through telephone contact or a programmed visit, the principal adverse clinical events as death, acute myocardial infarct, stent's thrombosis of the treated vessels or the occurence of a new revascularization, up to 6 months after the procedure.	The present manuscript is focused on the acute effect of GSH infusion on makers of oxidative stress and antioxidant status (H_2O_2 and 8-iso-PGF2alpha). The sample size was computed on the expected variation of oxidative stress markers based on previous published data (see page 9, lines 13-19 and reference 23). As reported in EudraCT website the clinical endpoints are listed among the secondary per protocol and we decided to not include them as the recruitment is still ongoing. Nevertheless, the study is unpowered for establishing an effect on clinical events after primary PCI.
2. The number of patients scheduled was 90, and they included 50. What about the sample size calculation ? What about this reduction to 50 ?	In methods section we included how the sample size was arrived at for the present interim analysis. We also provide reasons for the discrepancy noted by the reviewer (see page 9, lines 13-19). In the CONSORT diagram (see Figure 1) all the reasons to exclude patients screened were provided.
3. In the limitation section, authors write that their study is imitated by the lack of clinical end points and the small sample size. According to the original protocol, MACE were supposed to be an endpoint and the sample size was supposed to be twice higher. Could they comment?	As stated above, clinical endpoints were recorded as secondary endpoints, not as primary and the sample size was computed on oxidative stress markers changes after PCI (page 9, lines 13-19 and reference 23).
4. Authors describe that the AUC for troponin release was measured for each patient. Where are these values ? Troponins are supposed to be the primary endpoint surrogate, and they are not presented according to the method AUC).	We apologize for this mistake. We measured troponin with an enzyme immunoassay system as reported in the method section. Now we clarified this issue (see page 8, lines 13-15).
5. They introduce Table 1 and Table 2 by writing “Clinical and angiographic characteristics of patients are shown in Table 1 and 2. The baseline characteristics were well balanced between the two groups”. However, it appears that table 2 does not contain only baseline characteristics but also endpoints (cTFC, blush).	We apologize for the mistake and we removed inappropriate variables from Table 2.
6. Authors report a « tight correlation between percentage changes of H2O2 and cTpT levels from baseline to 5 days was found in treated group (Figure 2B) ». However, the r coefficient is less than 0.5 and accordingly, this relationship is only modest. Furthermore, they should perform a multivariate analysis taking into account the many other factors able to influence troponin release (vessel, delays, etc). I would also invert the X and Y axes.	We re-phrased to reflect the comment of the reviewer and we also modified the figure (see figure 3)

7. Apparently, an external Core Lab processed the data. Which corelab ?	The external Core Lab consists of 2 independent cardiologists that assessed the angiograms unaware of the study design (see page 7, lines 11-14).
8. Safety data (GSH) should also been included.	As reported in results section, no adverse events were observed in both GSH-treated and placebo groups (see page 10 lines 12-14).

Reviewer: 3

Make sure to follow consort criteria and rules; flowchart, etc.	We provided the CONSORT diagram and re-check the manuscript for consistency with CONSORT checklist (see figure 1 and supplied diagram attached).
The endpoints are written as hypothesis and not as endpoints.	As suggested, we revised the endpoints definition (see page 7, lines 22-26 and page 8, lines 1-2).
When referring to H2O2 levels, how do you know that its production? And if measured in peripheral blood, that it affected the heart?	We added reference about H_2O_2 sources (see page 4, lines 11-12). We measured H_2O_2 production as biomarker of oxidative stress that is associated with cardiovascular diseases.
Check accuracy of numbers. For instance: Table 1- killip class percentage incorrect.	We apologize for the mistake. We amended the table (see Table 1).
How do you define ischemia time.	We specified the definition of ischemia time (see Table 2)
Did all patients have occluded artery initially? Please provide initial TIMI flow and if there a difference between patients with occluded artery at baseline or not. Please assess difference between LAD and other IRA.	We clarified that all patients have occluded artery with TIMi Flow=0-1 (see page 10, lines 10-11).
Troponin- the description in text is not clear. The figure is more meaningful. But my understading that it was measured every 6h. Please add those time points.	As requested we added this time point (see page 10, lines 24-26; page 11, lines 3-5 and Figure 3).
There was no difference in echo results. Can glutathione affect troponin lab measurement without true effect on myocardial injury?	The paper is a pilot study and is aimed to explore whether intravenous GSH administration, just before acute reoxygenation of infarct myocardial areas and after wide recanalization, is able to blunt oxidative status thus reducing myocardial cell damage. It is conceivable that 5-days follow-up is too-short time to demonstrate LV recovery of function inside areas where stunned and hibernated myocardium coexist.

Myocardial function- There is no difference. Please don't call it a trend in the results section or discussion.	We removed as suggested. (See “myocardial function” subheading in results section).
Discussion- first paragraph too strong for the results.	We re-phrased the first paragraph (see page 11, lines 19-22).

Reviewer: 4

Section on Statistical Analysis - Replace "normal distribution of parameters" by "normal distribution of continuous variables"	Amended as suggested
Results section	
- Population, Table 1, Killip class, GSH group: It is 2 (8) not 2 (80).	We apologize for the mistake. We corrected the Table 1
- Table 3: For baseline data, the p-value is from a two-sample test. How were the p-value calculated for Reperfusion and Follow-up? Are really the mean values and standard deviations of interest? I would expect mean changes here that is calculating the difference from Reperfusion to Baseline for each patient and the computing mean and standard deviation and finally compare the mean changes of the two groups. I hope that the authors addressed the problem of possible unequal variances in the groups properly in the applied test. (This sentence is not only related to Table 3 but to all tests with continuous variables.)	Thanks for the suggestion, now we included the mean change from baseline after reperfusion (2h) and follow up (5d) (Table 3).
- Table 4, Follow-up, Placebo: Typo LVEF SD 3.2 or 3.0 but not 3.2.0	We apologize for the mistake. We amended the number accordingly.
-Table 4: I would first present the GSH group results than the placebo like in Tables 1 to 3. What is the meaning of * at P-value?	We changed the tables accordingly. We apologize for the typing error “*” in Tables, we deleted it.

VERSION 2 – REVIEW

REVIEWER	Shahar Lavi London Health Sciences Centre Western University London Ontario, Canada
REVIEW RETURNED	18-Jan-2019

GENERAL COMMENTS	The reviewer completed the checklist but made no further comments.

REVIEWER	Guido Knapp TU Dortmund University, Germany
REVIEW RETURNED	04-Feb-2019

GENERAL COMMENTS	The sample size calculation is done post-hoc. Starting with 157 posible patients, based on inclusion and7or exclusion criteria, the final number is 50 patients. In the sample size calculation, what does a mean difference of 20% mean?Usually you have to specify an absolute value Delta for the mean difference which should be detected. Please clarify!

VERSION 2 – AUTHOR RESPONSE

Reviewer: 3

I have no further comments

Answer: Thank you

Reviewer: 4

The sample size calculation is done post-hoc. Starting with 157 posible patients, based on inclusion and/or exclusion criteria, the final number is 50 patients. In the sample size calculation, what does a mean difference of 20% mean? Usually you have to specify an absolute value Delta for the mean difference which should be detected. Please clarify!

Answer: Thank you for your suggestion. We have rewritten the sample size to better clarify how it was calculated.