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Glutathione infusion before primary percutaneous coronary intervention: a randomized controlled trial.

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Complete List of Authors:	Tanzilli, Gaetano; University of Rome La Sapienza, Department of Heart and Great Vessels, Truscelli, Giovanni; Sapienza University of Rome Arrivi, Alessio; Department of Cardiology Carnevale, Roberto; Sapienza University, I Clinica Medica Placanica, Attilio; Department of Cardiology Viceconte, Nicola; Università degli studi di Roma "La Sapienza", Dipartimento Cuore e Grossi Vasi Raparelli, Valeria ; Sapienza University of Rome, I Clinica Medica, Department of Internal Medicine and Medical Specialties Mele, Rita; Sapienza University Cammisotto, Vittoria; Sapienza University, Department of Internal Medicine and Medical Specialties Nocella, Cristina; IRCCS NeuroMed Barillà, Francesco; Sapienza University , Department of the Heart and Great Vessels Lucisano, Luigi; Department of Cardiology Pennacchi, Mauro; Department of Cardiology Granatelli, Antonino; Department of Cardiology Dominici, Marcello; Department of Cardiology Basili, Stefania; Sapienza University of Rome, I Clinica Medica Gaudio, Carlo; University of Rome La Sapienza Mangeri, Enrico; Sapienza University , Department of the Heart and Great Vessels
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Manuscripts

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3 **Glutathione infusion before primary percutaneous coronary intervention: a randomized**
4 **controlled trial.**
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6 Gaetano Tanzilli¹, Giovanni Truscelli¹, Alessio Arrivi², Roberto Carnevale³, Attilio Placanica⁴,
7 Nicola Viceconte¹, Valeria Raparelli⁵, Rita Mele⁶, Vittoria Cammisotto⁷, Cristina Nocella⁸,
8 Francesco Barilla¹, Luigi Lucisano⁴, Mauro Pennacchi⁴, Antonino Granatelli⁴, Marcello Dominici²,
9 Stefania Basili⁵, Carlo Gaudio¹ and Enrico Mangieri¹
10
11
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14
15
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17

18 ¹ Department of the Heart and Great Vessels, Sapienza University of Rome, Rome, Italy
19

20 ² Department of Cardiology, "Santa Maria" Hospital, Terni, Italy
21

22 ³ Department of Medical-Surgical Sciences and Biotechnologies, Sapienza University, Latina, Italy
23

24 ⁴ Department of Cardiology, "San Giovanni Evangelista" Hospital, Tivoli, Italy
25

26 ⁵ Department of Experimental Medicine, Sapienza University of Rome, Rome, Italy
27

28 ⁶ Department of Surgical Sciences, Sapienza University, Rome, Italy
29

30 ⁷ Department of Internal Medicine and Medical Specialties, Sapienza University, Rome, Italy
31

32 ⁸ IRCCS NeuroMed, Pozzilli (IS), Italy
33
34
35
36
37

38 **Corresponding Author:**
39

40 Gaetano Tanzilli, MD, Department of Heart and Great Vessels "Attilio Reale", Sapienza University
41 of Rome, Viale del Policlinico 155, 00161 Rome, Italy; +39 06 49973240
42
43 gaetano.tanzilli@uniroma1.it
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Abstract

Objective In the setting of reperfused ST-elevation myocardial infarction (STEMI), increased production of reactive oxygen species (ROS) contributes to reperfusion injury. Among ROS, hydrogen peroxide (H_2O_2) showed toxic effects on human cardiomyocytes and may induce microcirculatory impairment. Glutathione(GSH) is a water-soluble tripeptide with a potent oxidant scavenging activity. We hypothesized that the infusion of GSH before acute reoxygenation might counteract the deleterious effects of increased H_2O_2 generation on myocardium.

Methods Fifty consecutive STEMI patients scheduled to undergo primary angioplasty were randomly assigned, before intervention, to receive an infusion of GSH (2500 mg/25ml over 10 min) followed by drug administration at the same doses at 24, 48, 72 hours elapsing time or placebo. Peripheral blood samples were obtained before and at the end of procedure as well as after 5 days. H_2O_2 production, 8-iso-PGF 2α formation, H_2O_2 breakdown activity (HBA) and nitric oxide (NO) bioavailability were determined. Serum cardiac-Troponin T (cTpT) was measured at admission and up to 5 days.

Results Following acute reperfusion, a significant reduction of H_2O_2 production ($p=0.0015$) and 8-isoPGF 2α levels ($p=0.0003$) as well as a significant increase in HBA ($p=0.000001$) and NO bioavailability ($p=0.035$) was found in the GSH group as compared with placebo. In treated patients, attenuated production of H_2O_2 persisted up to 5 days from the index procedure ($p=0.009$) and was linked to progressive decrease of cTpT levels ($r=0.41$, $p= 0.023$).

Conclusion The prophylactic and prolonged infusion of GSH determined a rapid onset and persistent blunting of H_2O_2 generation with positive benefits on myocardial cell survival.

Key words: Glutathione; STEMI; Reperfusion Injury; Reactive Oxygen Species; hydrogen peroxide.

Article summary

Strengths and limitations of this study

1. In patients who suffer from STEMI, acute reoxygenation of ischemic myocardium can induce additional myocardial cell injury mainly driven by heightened oxidative status.
2. Reactive oxygen species (ROS) generation further contributes to damage myocardium by limiting bioavailability of nitric oxide at microcirculatory level.
3. This pilot study demonstrates for the first time that in the setting of STEMI reperfusion the rapid onset and prolonged antioxidant (scavenging) activity obtained by infusion of GSH protects the myocardium.
4. This study is limited for the lack of clinical end points, the small sample size. Moreover, the absence of morphologic imaging, the qualitative evaluation of GSH-induced improvement of myocardial reperfusion indexes, as assessed in our study, might only represent the effect of a preserved microcirculatory responsiveness to vasoactive substances (i.e. NO) but unable to limit the expansion of myocardial cell damage.

Introduction

It is well known that reactive oxygen species (ROS) are produced at an accelerated rate in tissues subjected to reperfusion and that the accumulation of ROS contributes to reperfusion injury during reintroduction of molecular oxygen to the ischemic environment.[1,2]

In the setting of ST-Elevation Myocardial Infarction (STEMI), ROS-induced myocardial cell death occurs in the first few minutes of acute reoxygenation[3] and may continue for weeks to months by activation of apoptosis and autophagy processes.[4,5] ROS generation also contributes to structural capillary damage and endothelial dysfunction, which hinder the achievement of an optimal perfusion grade at microcirculatory level.[6,7] Over the time, this may result in adverse left ventricular (LV) remodeling and worse LV function.

Among ROS, hydrogen peroxide (H_2O_2) shows an important role in ischemia/reperfusion damage. In particular, the exposure of cultured human cardiomyocytes to H_2O_2 has determined rapid onset and progressive oxidative cell death.[8] Moreover, H_2O_2 influences platelet activation and promotes vascular dysfunction through thromboxane A_2 and isoprostanes formation, which are vasoconstrictors and powerful aggregating molecules derived from lipid peroxidation of esterified unsaturated fatty acids.[9-11]

Human possesses numerous enzymatic and non-enzymatic antioxidant systems. Among enzymatic system, glutathione peroxidase (GPx) plays an important role to prevent potentially deleterious effects of H_2O_2 . [12] Thus, the reduced plasma level of glutathione (GSH), a water-soluble tripeptide with a potent oxidant scavenging activity and fundamental substrate for GPx activity, could have a key role in promoting myocardial and endothelial cell damage.[13] In fact, a decrease in myocardial GSH content has been observed during ischemia and reperfusion of the ischemic myocardium.[14]

Despite robust evidences regarding the role of ROS in reperfusion injury, currently, in clinical practice, there are no treatments aimed to prevent ROS generation. According to our previous hypothesis,[15] during reperfusion of ST-elevation myocardial infarction (STEMI), the use of GSH

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3 might counteract deleterious effect of augmented oxidant activity. Therefore, we performed a pilot
4 *proof-of-concept* study to explore whether intravenous GSH administration, just before reopening of
5 infarct related artery and after effective reperfusion, is able to attenuate the cytotoxic activity of
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7 H₂O₂ on myocardium.
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10 11 **Methods**

12 13 **Study Design**

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15 GSH2014 (EurodraCT number 2014-004486-25) is a multicenter, no profit, randomized, double-
16 blind, prospective, placebo-controlled trial. The Department of Heart and Great Vessel "A. Reale",
17 Sapienza University of Rome, Italy was the coordinator centre. The study has been planned
18 according to principles of the declaration of Helsinki. Italian Medicines Agency (AIFA)
19 authorization and single Ethic Committee approval has been obtained for all the centers
20 participating the study (n=3). The coordinating center designated the protocol. An external Core
21 Lab processed the data. Written informed consent was obtained from all patients enrolled.
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30 31 **Study population and protocol**

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33 Between March and August 2017, we screened 157 consecutive STEMI patients, age >18 years,
34 both sexes, referred to the hospitals of the centre of Italy belonging to our working group for
35 primary percutaneous coronary intervention (pPCI). Inclusion criteria were: typical chest pain
36 lasting more than 30 min with pain onset <12 h, ST segment elevation >0.2 mV in at least two
37 contiguous leads in the initial ECG, successful pPCI (residual coronary stenosis <20%) and blood
38 sampling for biochemical determinations collected prior to pPCI. Exclusion criteria were:
39 symptoms duration > 12 h (n=15), rescue PCI (n=16), cardiogenic shock (n=3), left main disease
40 (n=3), evidence of coronary collateral vessels (Rentrop score of 2 or 3 for the area at risk) (n=5),
41 prior myocardial infarction (n=7), estimated glomerular filtration rate less than 30 ml/min (n=13),
42 acute infection (n=2), treatment with systemic corticosteroids (n=4) or oral anticoagulants (n=7),
43 malignancy (n=3), in-stent thrombosis (n=3), lack of consent to participate (n=18). Additionally, 8
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3 patients were ineligible because no blood samples were collected before the start of procedure.
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5 Finally, a total of 50 patients were enrolled.

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7 After percutaneous access was obtained, an intravenous bolus of 5.000 U of unfractionated heparin
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9 was administered, with sufficient supplements (if necessary) to maintain an activated clotting time
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11 (ACT) \geq 250 seconds during interventions. After baseline collection of peripheral blood samples,
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13 patients were randomized to an intravenous infusion of GSH (2500 mg/25 ml over 10 min) or
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15 placebo (saline solution) before pPCI. Patients underwent pPCI according to standard protocols.
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17 The use of thrombus aspiration, glycoprotein IIb/IIIa inhibition was left to the discretion of the
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19 treating physician. Multivessel PCI was performed in a staged fashion (7 to 10 days from index
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21 procedure). All patients had drug-eluting stents implanted in treated vessels. After interventions,
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23 GSH was infused at the same doses at 24, 48, 72 hours elapsing time. Further blood samples were
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25 obtained at the end of procedure and 5 days from index procedure. Serum cardiac Troponin T
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27 (cTpT) was measured at admission, before the procedure and after reperfusion every 6 h over the
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29 next 2 days, and thereafter once a day up to 5 days. Serum cTpT levels were measured using an
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31 automated enzyme immunoassay system (Thermo Scientific, code EH TNNT1) with the upper limit
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33 of normal being 0.035 ng/ml in our laboratory. The area under the curve (AUC) (expressed in
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35 arbitrary units) troponin release was measured in each patient by computerized planimetry. After
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37 60'-90', a post-procedural 12 leads- ECG for ST measurement were performed. Corrected TIMI
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39 frame count (cTFC) and TIMI myocardial perfusion grade (TMPG) were assessed before and after
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41 pPCI as previously described.¹⁶ The interventional cardiologists who performed angiographic
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43 evaluation were unaware of the study assignment. Digital angiograms were analyzed off-line with
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45 the use of an automated edge detection system (Cardiovascular Medical System, MEDIS Imaging
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47 Systems, Leiden, the Netherlands).

52 **Randomisation and blinding**

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54 An individual not involved in the study assigned codes to the study treatments, randomly allocated
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56 patients to an intravenous infusion of GSH (2500 mg/25 ml over 10 min) or placebo (saline
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3 solution) before pPCI and kept the key in a sealed envelope. The randomisation was carried out by a
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5 computer-generated random sequence. The authors and laboratory technicians were unaware of the
6
7 treatment allocation.

8 9 **Primary Endpoint**

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11 GSH prophylactic infusion at the time of pPCI followed by drug administration up to 3 days after
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13 procedure would attenuate ROS induced myocardial damage as assessed by measuring biochemical
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15 markers of cell death.

16 17 **Secondary Endpoints**

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19 GSH prophylactic infusion would improve myocardial reperfusion indexes as assessed by
20
21 evaluation of cTFC and TMPG.

22 23 **Peripheral blood samples**

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25 Blood samples were drawn from antecubital vein, before the start of procedure and after stent
26
27 deployment in all patients and then collected into tubes without anticoagulant or with 3.8% sodium
28
29 citrate, lithium heparin and EDTA and centrifuged at 300×g for 10 min to obtain supernatant.
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31 Plasma and serum aliquots were stored at -80°C in appropriate cuvettes until assayed. Markers of
32
33 oxidative stress and antioxidant system were analyzed in serum samples.

34 35 **H₂O₂ production**

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37 The H₂O₂ was evaluated by a Colorimetric Detection Kit (Arbor Assays, Ann Arbor, Michigan, US)
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39 and expressed as μM. Intra-assay and inter-assay coefficients of variation were 2.1% and 3.7%,
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41 respectively.
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44 45 **Determination of % HBA in peripheral serum**

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47 The evaluation of the ability to detoxify H₂O₂ was assessed by the analysis of the H₂O₂ breakdown
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49 activity (HBA) in serum with HBA assay kit (Aurogene, Rome, Italy, code HPSA-50). The % of
50
51 HBA was calculated according to the following formula: % Of HBA = [(Ac-As) / Ac] X 100 where
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53 Ac is the absorbance of H₂O₂ 1.4 mg/ml and As is the absorbance in the presence of the serum
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55 sample.
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Serum Nitric Oxide (NO) bioavailability

A colorimetric assay kit (Cell Biolabs, San Diego, CA US) was used to determine NO bioavailability by measurement of the nitric oxide metabolites nitrite and nitrate (NO_x) in the serum. Intra-assay and inter-assay coefficients of variation were 2.9% and 1.7% respectively.

Serum 8-iso-Prostaglandin F_{2α} formation

Concentration of 8-iso-PGF_{2α} in serum was measured by validated enzyme immunoassay (EIA) method (DRG International, Springfield, NJ, USA). Intra-assay and inter-assay coefficients of variation were 5.8% and 5.0% respectively. Values were expressed as pmol/L.

Myocardial function

After 120 minutes and 5 days from the intervention, left ventricular end-diastolic volume (LVEDV), left ventricular end-systolic volume (LVESV) and ejection fraction (LVEF) were calculated by the biplane Simpson's rule, as recommended by the American Society of Echocardiography. The mean values of three measurements were used for statistical evaluation.

Statistical analysis

Categorical variables were reported as counts (percentage) and continuous variables as means ± standard deviation (SD). We tested the independence of categorical variables by χ^2 test and the normal distribution of parameters by Kolmogorov-Smirnov test. We used Student paired and unpaired t test, repeated measure ANOVA and Pearson product-moment correlation analysis to evaluate normally distributed continuous variables. Appropriate nonparametric tests (Mann-Whitney U test, Wilcoxon rank test and Spearman rank correlation test) were employed for all the other variables. As an overall nonparametric ANOVA, the Friedman test for the analysis of intragroup variations was used. In cases of significance, we compared pair related samples using the Wilcoxon test. The intergroup analysis was performed with the nonparametric Mann-Whitney U-test. Only two-tailed probabilities were used for testing statistical significance. Probability values <

0.05 were regarded as statistically significant. All calculations were made with the computer program STATISTICA 7 (StatSoft, Tulsa, OK, USA).

Results

Population. Twenty-five patients randomly received GSH and 25 placebo. All patients completed the phases of the study and no side effects were observed during or after GSH or placebo infusion. Clinical and angiographic characteristics of patients are shown in Table 1 and 2. The baseline characteristics were well balanced between the two groups.

Oxidative stress, antioxidant status and vascular function in peripheral samples. Biochemical data are summarized in Table 3. Baseline H₂O₂ and 8-iso-PGF2 α levels were similar between treated patients and controls. After PCI, a significant reduction of H₂O₂ production and 8-iso-PGF2 α levels was observed in GSH group as compared to controls (Figure 1A and B). Moreover, a significant increase in HBA and NO bioavailability was observed (Figure 1C and D). At the 5 days from index procedure, a persistent significant reduction of H₂O₂ production and a sustained increase in HBA and NO bioavailability was observed in the GSH group as compared with controls (Figure 1A-D).

Serological markers of myocardial injury. Baseline cTnT mean values were similar between GSH and placebo groups (176.0 ± 20.9 pg/ml vs. 165.4 ± 20.9 pg/ml, $p=0.079$). At 12 hours and 5 days after pPCI, GSH-treated patients showed a progressive decrease of cTnT levels (170.0 ± 44.7 pg/ml and 137.9 ± 23.7 pg/ml; $-21 \pm 23.1\%$, $p=0.009$ vs. baseline). Differently, a significant increase and persistence of high values of cTnT were observed in placebo group (183.0 ± 34.8 pg/ml and 181.9 ± 18.0 pg/ml; $+12.4 \pm 23.1\%$, $p=0.029$ vs. baseline) (Figure 2A). A tight correlation between percentage changes of H₂O₂ and cTnT levels from baseline to 5 days was found in treated group (Figure 2B).

Myocardial Reperfusion indexes. Post procedural cTFC values did not show a statistical significant reduction between treated and control groups (20.7 ± 7.3 vs. 23.4 ± 5.1 , $p=0.156$). Interestingly, 6 patients (24%) in the placebo group and 15 (60%) patients in GSH group reached

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3 lower-risk (≤ 20 frames/s) cTFC class ($p=0.019$). After PCI, $\text{TMPG} \geq 2$ was assessed in 21 patients
4 (84%) and 14 patients (56%) of the GSH and placebo groups, respectively ($p = 0.064$). Of note, 11
5 patients (44%) of the GSH group only had $\text{TMPG}=3$ ($p=0.0002$ vs. controls). Post-reperfusion
6 cTFC values showed a significant correlation with changes of 8-iso-PGF 2α ($R=0.55$, $p=0.012$)
7 levels from baseline.
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13 **Myocardial function.** Myocardial function was not different between groups after either baseline
14 or at discharge. There was no significant difference between groups regarding LVEF, LVEDV or
15 LVESV at any time point, although a trend towards reduced LVED, index of left ventricular
16 remodelling, was assessed in treated patients (Table 4).
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21 Discussion

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24 This pilot study demonstrates for the first time that in the setting of STEMI reperfusion the rapid
25 onset and prolonged antioxidant (scavenging) activity obtained by infusion of GSH protects the
26 myocardium. Data from experimental and clinical studies suggest that following reperfusion
27 myocardial cells death largely contributes to the final infarct size.[17,18] On the other hand, the
28 extent of damaged myocardium is the most important predictor of adverse ventricular remodelling
29 and it is linearly dependent upon the amount of myocardial salvage by and after reperfusion. Thus,
30 attenuation of pro-oxidant state is an important goal in cardioprotective interventions.[19]
31 Noteworthy, the serum of GSH treated patients showed a greater capacity to detoxify H_2O_2
32 evaluated by the HBA, an assay that measure the percentage of H_2O_2 neutralized into the samples.²⁰
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34 We found an early and considerable increase of HBA, with positive effects on myocardial cell
35 survival. Current evidences demonstrate that hostile oxidant environment promotes cardiomyocyte
36 death in the first few minutes of reflow suggesting the existence of a tight window of effective
37 cardioprotection.[21,22] Therefore, ROS-induced injury may continue for weeks to months as a
38 result of activation of programmed cell death. Our data have shown a persistent heightened
39 oxidative status along with decreased scavenging activity in untreated patients. This behaviour
40 makes the duration of pharmacologic interventions a central point of cardioprotection strategies. In
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3 the present study, GSH infusion, starting just before reperfusion with subsequent administration up
4 to 3 days after, promoted early and sustained increase of serum HBA with attenuated production of
5 H_2O_2 which was highly related to progressive significant reduction of serological signs of
6 myocardial injury. In the clinical setting, the efficacy of reperfusion relies on the assessment of
7 cardiac biomarkers release, which represents the demonstration of irreversible decay of cardiac
8 myocytes. Our data show a progressive significant decrease of serum cTnT release during the 5
9 days of reperfusion in the GSH-treated patients compared with the control group resulting in a 21%
10 reduction of myocardial damage. Despite that, in our population, the systolic function was not
11 different between groups after reperfusion, although a trend towards reduced LVEDV was observed
12 in treated patients. A possible explanation relies on the fact that inside the area at risk variable
13 amount of hibernated and stunned myocardium may coexist, thus affecting the prompt recovery of
14 contractility after reperfusion.[23] Cells have a number of mechanisms for dealing with the toxic
15 effects of oxygen. One of the most important is connected with the widely distributed tripeptide
16 thiol glutathione.[12,24] In particular, the glutathione redox cycle is a more efficient antioxidant
17 protective mechanism of the heart, which acts by maintaining thiol groups of enzymes and other
18 proteins in their reduced state thus preventing cell membrane lipid peroxidation and limiting
19 cardiomyocytes loss.[25] Furthermore, in our study, a close relation between reduced myocardial
20 reperfusion perfusion, increased of 8-iso-PGF 2α serum levels has been observed, suggesting that
21 oxidative unbalance may be involved in microcirculation functional damage. As previously
22 reported, impaired tissue-level perfusion develops within minutes of established acute
23 revascularization of ischemic areas[26] and persists for at least 1 week.[27] In this context, there is
24 robust evidence that ROS mediated isoprostanes production contributes importantly to the post-
25 reperfusion microvascular impairment.[28,29] Current findings implement this observation by
26 demonstrating a sustained production of isoprostanes up to 5 days after reperfusion thereby
27 suggesting their contributory role in the pathogenesis and persistence of microvascular dysfunction
28 that may affect myocardial cell survival. The infusion of GSH before and 24, 48, 72 hours after
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3 pPCI reduced isoprostanes serum levels and their reduction was linked to improvement of
4 myocardial reperfusion indexes. Moreover, the increase in extracellular peroxide oxidants may
5 reduce bioavailability of nitric oxide that is thought to contribute to promoting platelet hyperactivity
6 and vasoconstriction.[9] In our study, GSH supplementation seems to have a primary role in
7 preserving NO bioavailability and its vasodilator capacity at microcirculatory level.
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13 This study has implications and limitations. Although the positive effects on reperfusion indexes
14 and biochemical signs of myocardial necrosis suggest the value of prophylactic and prolonged GSH
15 administration in preventing reperfusion injury, the lack of clinical end points and the small sample
16 size limit the readiness of the study for clinical purposes. In addition, at discharge LVEF did not
17 change between treated patients and controls thereby limiting the possibility to translate
18 biochemical and angiographic benefits into improvement of prognosis.
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26 A further concern regards the lack of quantitative assessment of both infarct size and microvascular
27 obstruction extent. Within a defined area at risk, the manifestations of ischemia-reperfusion
28 vascular injury go from reversible functional impairments to irreversible structural damage and
29 contribute to final amount of infarct myocardium. In absence of morphologic imaging, the
30 qualitative evaluation of GSH-induced improvement of myocardial reperfusion indexes, as assessed
31 in our study, might only represent the effect of a preserved microcirculatory responsiveness to
32 vasoactive substances (i.e. NO) but unable to limit the expansion of myocardial cell damage.
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3 In conclusion, we have shown that prophylactic and prolonged GSH infusion mitigates the negative
4 effects of the excessive and persistent H₂O₂ formation on myocardial cells. Therefore, in patients
5 undergoing pPCI the infusion of a powerful antioxidant scavenger, such as GSH, may be useful to
6 improve microcirculatory perfusion in order to further blunt the injury of myocardial cells. At
7 moment, our data represent only a hypothesis generating observation that requires larger STEMI
8 population and prolonged follow-up to confirm the role of GSH administration as cardioprotective
9 therapy.
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Contributors

GT, EM led on the conception, design and writing of the study and study protocol with substantial contributions to the design, writing, critical review of intellectual content; GT, AA, AP, NV, RM, enrollment of patients; RC, VC, CN, provided laboratory analyses; VR, SB, provided further essential statistical advice and expertise on the study protocol; FB, LL, MP, CG, providing expert clinical support; AG, MD, made substantial contributions to the trial design and management.

Ethics and dissemination

Italian Medicines Agency (AIFA) authorization and single Ethic Committee approval has been obtained for all the centers participating the study. Dissemination of results will be via peer-reviewed research publications both online and in print, conference presentations, posters, patient forums and Trust bulletins.

Data Availability

The data used to support the findings of this study are included within the article.

A data sharing statement

No additional data available

Conflicts of Interest

Authors declare that there are not conflicts of interest.

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Table 1. Clinical characteristics of the study population

Variables	GSH group (n=25)	Placebo group (n=25)	P value
Age (y, mean±SD)	66 ± 10.7	66.9 ± 9.1	0.74
Male, n (%)	15 (60)	13 (52)	0.98
Body-mass index§ (mean+SD)	26.9 ± 3.9	20 ± 3.8	0.38
Killip class ≥ 3, n (%)	2 (8)	0 (0)	0.47
Diabetes Mellitus, n (%)	5 (20)	5 (20)	1
Hypertension, n (%)	14 (56)	17 (68)	0.56
Dyslipidemia, n (%)	11 (44)	13 (52)	0.77
Statin use, n (%)	8 (32)	8 (32)	1
Smokers, n (%)	17 (68)	13 (52)	0.38

§ The body-mass index is the weight in kilograms divided by the square of the height in meters.

Table 2. Angiographic parameters

Variables	GSH group (n=25)	Placebo group (n=25)	P value
Ischemia time (min; mean \pm SD)	286 \pm 88	270 \pm 96	0.85
Thrombus Burden \geq 3, n (%)	12 (48)	11 (44)	0.77
Thrombus aspiration, n (%)	13 (52)	12 (48)	0.87
GP IIb/IIIa inhibitors, n (%)	2 (8)	3 (12)	0.63
MVD, n (%)	13 (52)	11 (44)	0.77
2 vessels,	8 (32)	5 (20)	0.89
3 vessels,	5 (20)	6 (24)	
Staged PCI, n (%)	9 (36)	5 (20)	
IRA:			
LAD, n (%)	10 (40)	9 (36)	0.77
LCx, n (%)	5 (20)	6 (24)	0.73
RCA, n (%)	10 (40)	10 (40)	1
cTFC after PCI (frames/sec, mean \pm SD)	20.7 \pm 7.3	23.4 \pm 5.1	0.156
cTFC < 20 frames/sec, n (%)	15 (60)	6 (24)	0.019
MPG after PCI \geq 2, n (%)	21 (64)	14 (48)	0.064

MPG after PCI = 3, n 11 (44) 0 (0) 0.002
(%)

PCI = percutaneous coronary intervention; IRA = infarct related coronary artery; LAD = left anterior descending coronary artery; LCx = left circumflex coronary artery; RCA = Right coronary artery; MPG = myocardial perfusion grade; cTFC = corrected TIMI frame count.

Table 3. Biochemical data

Variable	Baseline			Reperfusion			Follow-up (5 days)		
	GSH	Placebo	p	GSH	Placebo	p	GSH	Placebo	p
H₂O₂ μM, mean±SD	40.6±8.4	43.6±11.6	0.305	28.4±12	42.8±14.1	0.0003	24±7	39.5±17.3	0.0001
8-iso-PGF2α pmol/L, mean±SD	214.6±81.1	211.9±92.1	0.91	163.6±44.7	217.6±51.6	0.0003	159.9±34.2	213.1±50.9	0.0001
HBA %, mean±SD	43.6±7.4	43.4±11.9	0.94	57.9±8.6	43.9±8.7	0.0001	62.9±10.5	45.2±13.0	0.0001
NO μM, mean±SD	16.3±5.7	16.5±4.7	0.89	27.7±7.2	22.4±10	0.0356	35.5±8.1	23.5±15.5	0.0013

GSH = reduced Glutathione; H₂O₂ = Hydrogen Peroxide; 8-iso-PGF2α = 8-iso-Prostaglandin F2α;
HBA = H₂O₂ break-down activity

Table 4. Left Ventricular echocardiographic parameters at baseline and at follow-up

Echo parameters	Placebo (n= 25)	GSH (n=25)	<i>P</i> -value*
Baseline			
LVEDV (mL/m²)	121.3 ± 17.2	124.4 ± 22.3	0.44
LVESV (mL/m²)	65.4 ± 11.3	66.3 ± 13.2	0.91
LVEF (%)	47.5 ± 4.9	46.9 ± 4.8	0.42
Follow-up			
LVEDV (mL/m²)	118.1 ± 17.8	113.2 ± 14.1	0.42
LVESV (mL/m²)	60.9 ± 10.7	58.8 ± 12.5	0.91
LVEF (%)	49.1 ± 3.2.0	49.8 ± 3.7	0.42
LVEDV = left ventricular end-diastolic volume; LVESV = left ventricular end-Systolic volume; LVEF = left ventricular ejection fraction-diastolic volume			

Figure Legend

Figure 1. H₂O₂ production (A), 8-iso-PGF₂α formation (B), hydrogen peroxide breakdown activity (HBA) (C) and NO bioavailability (D) at baseline, after 2 hours (T2h) and at the 5 days (T5d) from the PCI in patients received GSH (n=25, dashed line) or placebo (n=25, continuous line) (**p <0.01, ***p <0.001, *p <0.05).

Figure 2. cTnT levels at baseline, after 12 hours (T12h) and at the 5 days (T5d) from the PCI in patients received GSH (n=25, dashed line) or placebo (n=25, continuous line) (A) (**p <0.01 vs. T0, *p <0.05 vs. T0, §p <0.05 between groups).

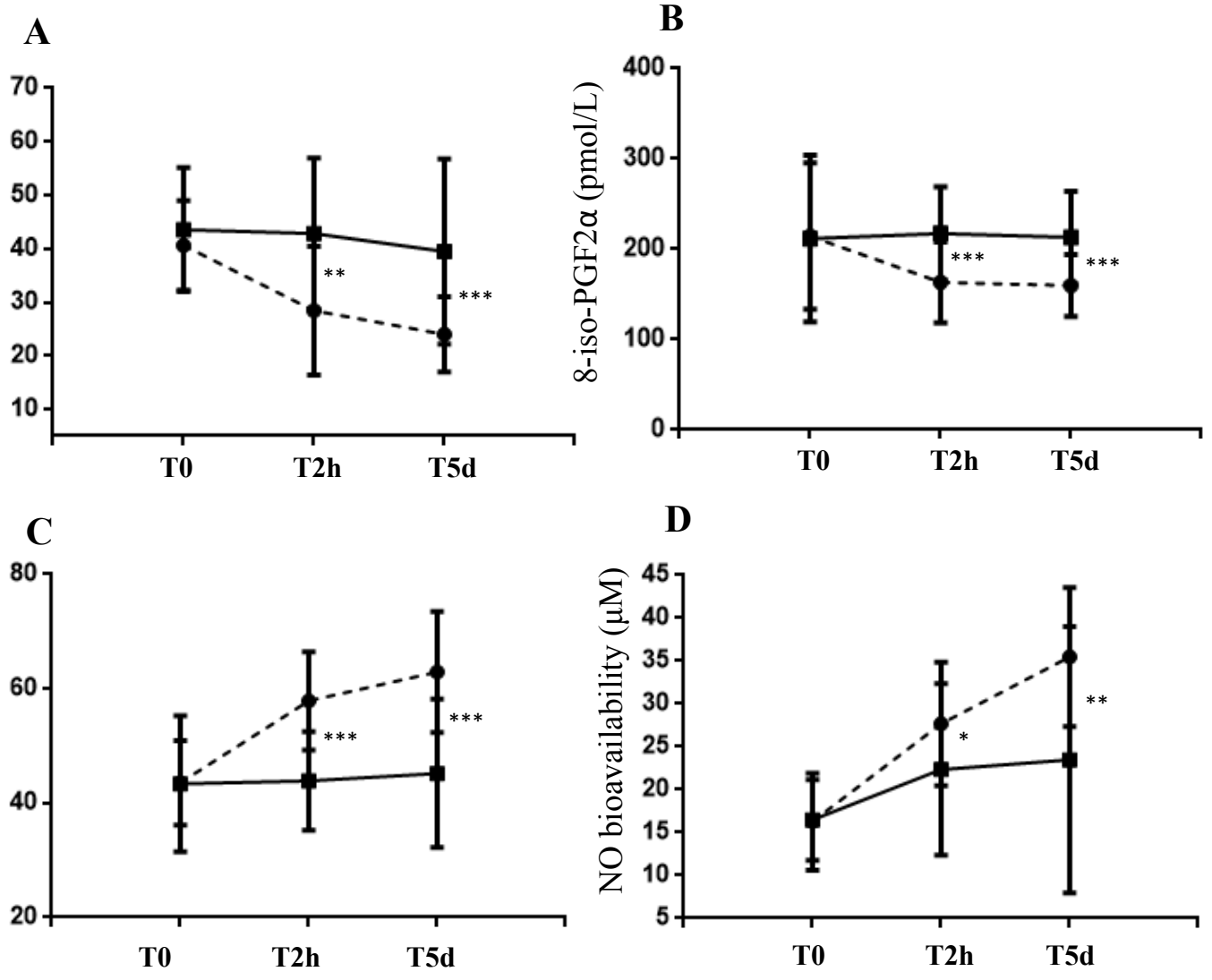
Linear correlation between % Δ H₂O₂ and % Δ cTnT in GSH treated group (B).

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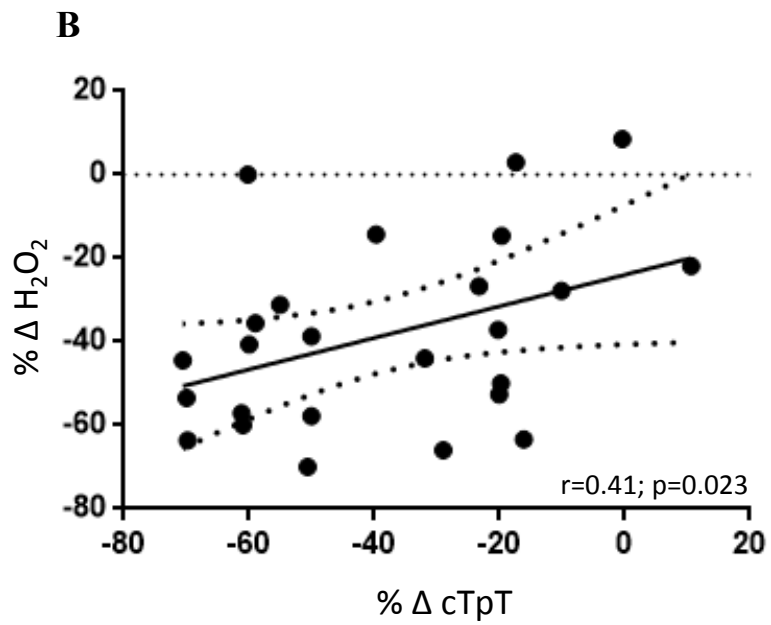
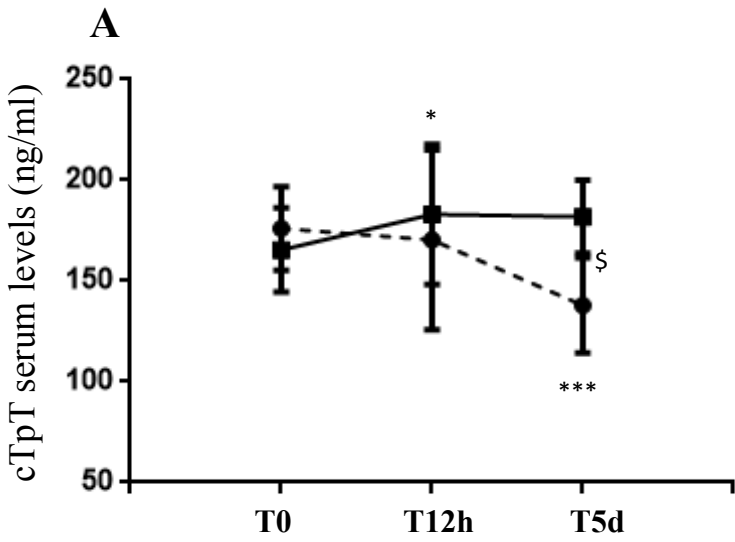
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GSH Treatment
 Placebo Treatment

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●--- GSH Treatment
■--- Placebo Treatment





CONSORT 2010 checklist of information to include when reporting a randomised trial*

Section/Topic	Item No	Checklist item	Reported on page No
Title and abstract			
	1a	Identification as a randomised trial in the title	1
	1b	Structured summary of trial design, methods, results, and conclusions (for specific guidance see CONSORT for abstracts)	2
Introduction			
Background and objectives	2a	Scientific background and explanation of rationale	4
	2b	Specific objectives or hypotheses	5
Methods			
Trial design	3a	Description of trial design (such as parallel, factorial) including allocation ratio	6
	3b	Important changes to methods after trial commencement (such as eligibility criteria), with reasons	NA
Participants	4a	Eligibility criteria for participants	5
	4b	Settings and locations where the data were collected	5
Interventions	5	The interventions for each group with sufficient details to allow replication, including how and when they were actually administered	6
Outcomes	6a	Completely defined pre-specified primary and secondary outcome measures, including how and when they were assessed	6
	6b	Any changes to trial outcomes after the trial commenced, with reasons	NA
Sample size	7a	How sample size was determined	NA
	7b	When applicable, explanation of any interim analyses and stopping guidelines	NA
Randomisation:			
Sequence generation	8a	Method used to generate the random allocation sequence	6-7
	8b	Type of randomisation; details of any restriction (such as blocking and block size)	6-7
Allocation concealment mechanism	9	Mechanism used to implement the random allocation sequence (such as sequentially numbered containers), describing any steps taken to conceal the sequence until interventions were assigned	NA
Implementation	10	Who generated the random allocation sequence, who enrolled participants, and who assigned participants to interventions	6-7
Blinding	11a	If done, who was blinded after assignment to interventions (for example, participants, care providers, those	6-7

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2		assessing outcomes) and how	
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4		11b If relevant, description of the similarity of interventions	NA
5	Statistical methods	12a Statistical methods used to compare groups for primary and secondary outcomes	8
6		12b Methods for additional analyses, such as subgroup analyses and adjusted analyses	8
7			
8	Results		
9	Participant flow (a	13a For each group, the numbers of participants who were randomly assigned, received intended treatment, and	8
10	diagram is strongly	were analysed for the primary outcome	
11	recommended)	13b For each group, losses and exclusions after randomisation, together with reasons	NA
12	Recruitment	14a Dates defining the periods of recruitment and follow-up	5
13		14b Why the trial ended or was stopped	NA
14	Baseline data	15 A table showing baseline demographic and clinical characteristics for each group	8 and Table 1
15	Numbers analysed	16 For each group, number of participants (denominator) included in each analysis and whether the analysis was	8
16		by original assigned groups	
17			
18	Outcomes and	17a For each primary and secondary outcome, results for each group, and the estimated effect size and its	8 and 9
19	estimation	precision (such as 95% confidence interval)	
20		17b For binary outcomes, presentation of both absolute and relative effect sizes is recommended	8 and 9
21	Ancillary analyses	18 Results of any other analyses performed, including subgroup analyses and adjusted analyses, distinguishing	NA
22		pre-specified from exploratory	
23			
24	Harms	19 All important harms or unintended effects in each group (for specific guidance see CONSORT for harms)	NA
25			
26	Discussion		
27	Limitations	20 Trial limitations, addressing sources of potential bias, imprecision, and, if relevant, multiplicity of analyses	11
28	Generalisability	21 Generalisability (external validity, applicability) of the trial findings	12
29	Interpretation	22 Interpretation consistent with results, balancing benefits and harms, and considering other relevant evidence	9-12
30			
31	Other information		
32	Registration	23 Registration number and name of trial registry	5
33	Protocol	24 Where the full trial protocol can be accessed, if available	5
34	Funding	25 Sources of funding and other support (such as supply of drugs), role of funders	NA
35			

37 *We strongly recommend reading this statement in conjunction with the CONSORT 2010 Explanation and Elaboration for important clarifications on all the items. If relevant, we also
 38 recommend reading CONSORT extensions for cluster randomised trials, non-inferiority and equivalence trials, non-pharmacological treatments, herbal interventions, and pragmatic trials.
 39 Additional extensions are forthcoming: for those and for up to date references relevant to this checklist, see www.consort-statement.org.

BMJ Open

Glutathione infusion before primary percutaneous coronary intervention: a randomized controlled pilot study.

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Complete List of Authors:	Tanzilli, Gaetano; University of Rome La Sapienza, Department of Heart and Great Vessels, Truscelli, Giovanni; Sapienza University of Rome Arrivi, Alessio; Department of Cardiology Carnevale, Roberto; Sapienza University, I Clinica Medica Placanica, Attilio; Department of Cardiology Viceconte, Nicola; Università degli studi di Roma "La Sapienza", Dipartimento Cuore e Grossi Vasi Raparelli, Valeria ; Sapienza University of Rome, I Clinica Medica, Department of Internal Medicine and Medical Specialties Mele, Rita; Sapienza University Cammisotto, Vittoria; Sapienza University, Department of Internal Medicine and Medical Specialties Nocella, Cristina; IRCCS NeuroMed Barillà, Francesco; Sapienza University , Department of the Heart and Great Vessels Lucisano, Luigi; Department of Cardiology Pennacchi, Mauro; Department of Cardiology Granatelli, Antonino; Department of Cardiology Dominici, Marcello; Department of Cardiology Basili, Stefania; Sapienza University of Rome, I Clinica Medica Gaudio, Carlo; University of Rome La Sapienza Mangeri, Enrico; Sapienza University , Department of the Heart and Great Vessels
Primary Subject Heading:	Cardiovascular medicine
Secondary Subject Heading:	Pharmacology and therapeutics
Keywords:	Glutathione, Reperfusion Injury, STEMI, hydrogen peroxide

SCHOLARONE™
Manuscripts

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3 1 **Glutathione infusion before primary percutaneous coronary intervention: a randomized**
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5 2 **controlled pilot study.**

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8 3 Gaetano Tanzilli¹, Giovanni Truscelli¹, Alessio Arrivi², Roberto Carnevale³, Attilio Placanica⁴,
9
10 4 Nicola Viceconte¹, Valeria Raparelli⁵, Rita Mele⁶, Vittoria Cammisotto⁷, Cristina Nocella⁸,
11
12 5 Francesco Barillà¹, Luigi Lucisano⁴, Mauro Pennacchi⁴, Antonino Granatelli⁴, Marcello Dominici²,
13
14 6 Stefania Basili⁵, Carlo Gaudio¹ and Enrico Mangieri¹

15
16
17 7
18
19 8 ¹ Department of the Heart and Great Vessels, Sapienza University of Rome, Rome, Italy

20
21 9 ² Department of Cardiology, "Santa Maria" Hospital, Terni, Italy

22
23
24 10 ³ Department of Medical-Surgical Sciences and Biotechnologies, Sapienza University, Latina, Italy

25
26 11 ⁴ Department of Cardiology, "San Giovanni Evangelista" Hospital, Tivoli, Italy

27
28 12 ⁵ Department of Experimental Medicine, Sapienza University of Rome, Rome, Italy

29
30 13 ⁶ Department of Surgical Sciences, Sapienza University, Rome, Italy

31
32
33 14 ⁷ Department of Internal Medicine and Medical Specialties, Sapienza University, Rome, Italy

34
35 15 ⁸ IRCCS NeuroMed, Pozzilli (IS), Italy

36
37
38 16
39
40 17 **Corresponding Author:**

41
42
43 18 Gaetano Tanzilli, MD, Department of Heart and Great Vessels "Attilio Reale", Sapienza University
44
45 19 of Rome, Viale del Policlinico 155, 00161 Rome, Italy; +39 06 49973240

46
47 20 gaetano.tanzilli@uniroma1.it

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1 **Abstract**

2 **Objective-** In the setting of reperfused ST-elevation myocardial infarction (STEMI), increased
3 production of reactive oxygen species (ROS) contributes to reperfusion injury. Among ROS,
4 hydrogen peroxide (H₂O₂) showed toxic effects on human cardiomyocytes and may induce
5 microcirculatory impairment. Glutathione (GSH) is a water-soluble tripeptide with a potent oxidant
6 scavenging activity. We hypothesized that the infusion of GSH before acute reoxygenation might
7 counteract the deleterious effects of increased H₂O₂ generation on myocardium.

8 **Methods-** Fifty consecutive STEMI patients scheduled to undergo primary angioplasty were
9 randomly assigned, before intervention, to receive an infusion of GSH (2500 mg/25ml over 10 min)
10 followed by drug administration at the same doses at 24, 48, 72 hours elapsing time or placebo.
11 Peripheral blood samples were obtained before and at the end of procedure as well as after 5 days.
12 H₂O₂ production, 8-iso-PGF₂α formation, H₂O₂ breakdown activity (HBA) and nitric oxide (NO)
13 bioavailability were determined. Serum cardiac-Troponin T (cTpT) was measured at admission and
14 up to 5 days.

15 **Results-** Following acute reperfusion, a significant reduction of H₂O₂ production (p=0.0015) and 8-
16 iso-PGF₂α levels (p=0.0003) as well as a significant increase in HBA (p<0.0001) and NO
17 bioavailability (p=0.035) was found in the GSH group as compared with placebo. In treated
18 patients, attenuated production of H₂O₂ persisted up to 5 days from the index procedure (p=0.009)
19 and these changes were linked to those of cTpT levels (r=0.41, p= 0.023).

20 **Conclusion** The prophylactic and prolonged infusion of GSH seems determined a rapid onset and
21 persistent blunting of H₂O₂ generation improving myocardial cell survival. Nevertheless, a larger
22 trial, adequately powered for evaluation of clinical endpoints, is ongoing to confirm the current
23 finding.

24
25 **Key words:** Glutathione; STEMI; Reperfusion Injury; Reactive Oxygen Species; hydrogen
26 peroxide, Percutaneous Coronary Interventions.

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3 1 **Article summary**
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5 2 **Strengths and limitations of this study**
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8 3 1. In patients who suffer from STEMI, acute reoxygenation of ischemic myocardium can
9 induce additional myocardial cell injury mainly driven by heightened oxidative status.
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12 5 2. Reactive oxygen species (ROS) generation further contributes to damage myocardium by
13 limiting bioavailability of nitric oxide at microcirculatory level.
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17 7 3. This pilot study demonstrates that in the setting of STEMI reperfusion the rapid onset and
18 prolonged antioxidant (scavenging) activity obtained by infusion of glutathione (GSH)
19 8 protects the myocardium.
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24 10 4. This study is limited by the lack of clinical end points and the small sample size. Moreover,
25 qualitative assessment of GSH-induced improvement of myocardial reperfusion indexes,
26 11 might only represent the effect of a preserved microcirculatory responsiveness to vasoactive
27 12 substances (i.e. NO) but unable to limit the expansion of myocardial cell damage.
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1 **Introduction**

2 It is well known that reactive oxygen species (ROS) are produced at an accelerated rate in tissues
3 subjected to reperfusion and that the accumulation of ROS contributes to reperfusion injury during
4 reintroduction of molecular oxygen to the ischemic environment.[1,2]

5 In the setting of ST-elevation myocardial infarction (STEMI), ROS-induced myocardial cell death
6 occurs in the first few minutes of acute reoxygenation[3] and may continue for weeks to months by
7 activation of apoptosis and autophagy processes.[4,5] ROS generation also contributes to structural
8 capillary damage and endothelial dysfunction, which hinder the achievement of an optimal
9 perfusion grade at microcirculatory level.[6,7] Over the time, this may result in adverse left
10 ventricular (LV) remodeling and worse LV function.[8-10]

11 Among ROS, hydrogen peroxide (H_2O_2) is produced by many enzymes including for example
12 xanthine oxidase, lipoxygenase and, in particular, NADPH oxidase.[11] H_2O_2 shows an important
13 role in ischemia/reperfusion damage. In particular, the exposure of cultured human cardiomyocytes
14 to H_2O_2 has determined rapid onset and progressive oxidative cell death.[12] Moreover, H_2O_2
15 influences platelet activation and promotes vascular dysfunction through thromboxane A_2 and
16 isoprostanes formation, which are vasoconstrictors and powerful aggregating molecules derived
17 from lipid peroxidation of esterified unsaturated fatty acids.[13-15]

18 Human possesses numerous enzymatic and non-enzymatic antioxidant systems. Among enzymatic
19 system, glutathione peroxidase (GPx) plays an important role to prevent potentially deleterious
20 effects of H_2O_2 . [16] Thus, the reduced plasma level of glutathione (GSH), a water-soluble tripeptide
21 with a potent oxidant scavenging activity and fundamental substrate for GPx activity, could have a
22 key role in promoting myocardial and endothelial cell damage.[17] In fact, a decrease in myocardial
23 GSH content has been observed during ischemia and reperfusion of the ischemic myocardium.[18]
24 Despite robust evidences regarding the role of ROS in reperfusion injury, currently, in clinical
25 practice, there are no treatments aimed at preventing ROS generation.

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1 Preclinical study of ischemia/reperfusion demonstrated that timely application of GSH provides
2 better cardio-protection at higher doses [19]. Our hypothesis is that the use of GSH might
3 counteract deleterious effect of augmented oxidant activity during reperfusion of STEMI [20].
4 Currently, a glutathione solution is available for intravenous usage to reduce side effects of
5 chemotherapy treatment for cancer with a tolerable safety profile, however it has never been tested
6 in the setting of patients with STEMI.
7 Therefore, we performed a pilot study to explore whether a short-term intravenous GSH
8 administration, just before and after a primary percutaneous coronary intervention (p-PCI) in
9 STEMI patients, was able to reduce oxidative stress and antioxidant status markers, resulting in a
10 reduction of the myocardial damage.

12 **Methods**

13 **Study Design**

14 GSH2014 is a multicenter, no profit, randomized, double-blind, prospective, placebo-controlled
15 trial. The Department of Heart and Great Vessel "A. Reale", Sapienza University of Rome, Italy
16 was the coordinator center and designed the protocol (see Supplementary file). Two other centers,
17 "Santa Maria" Terni Hospital and "San Giovanni Evangelista" Tivoli Hospital, both in Italy, were
18 involved in the study as recruiting site.

19 The study has been planned according to principles of the declaration of Helsinki. Ethic Committee
20 of the coordinator centre and Italian Medicines Agency (AIFA) (Date of Competent Authority
21 Decision: 2015-01-13) authorized the study. Written informed consent was obtained from all
22 patients enrolled. (<https://www.clinicaltrialsregister.eu/ctr-search/trial/2014-004486-25/IT#N>)

23 **Patient and Public Involvement**

24 Patients and or public were not involved in the different stages of the study (including the design
25 and the recruitment phase). However, we intend to disseminate the main results to trial participants

1 and will seek patient and public involvement in the development of an appropriate method of
2 dissemination.

3 **Study population and protocol**

4 Between March and August 2017, 157 consecutive STEMI patients, age >18 years, both sexes,
5 referred to the three enrolling centers for primary percutaneous coronary intervention (p-PCI) were
6 screened to enter in the study. Inclusion criteria were: typical chest pain lasting more than 30 min
7 with pain onset <12 h, ST segment elevation >0.2 mV in at least two contiguous leads in the initial
8 ECG, successful p-PCI (residual coronary stenosis <20%) and blood sampling for biochemical
9 determinations collected prior to p-PCI.

10 Exclusion criteria were: symptoms duration > 12 h (n=15), rescue PCI (n=16), cardiogenic shock
11 (n=3), left main disease (n=3), evidence of coronary collateral vessels (Rentrop score of 2 or 3 for
12 the area at risk) (n=5), prior myocardial infarction (n=7), estimated glomerular filtration rate less
13 than 30 ml/min (n=13), acute infection (n=2), treatment with systemic corticosteroids (n=4) or oral
14 anticoagulants (n=7), malignancy (n=3), in-stent thrombosis (n=3), lack of consent to participate
15 (n=18). Additionally, 8 patients were ineligible because no blood samples were collected before the
16 start of procedure. Finally, a total of 50 patients were enrolled (see Figure 1- CONSORT diagram).
17 The present analysis reported the results of the interim analysis (pre-planned in the protocol) on the
18 acute effect of GSH infusion on markers of oxidative stress.

19 After percutaneous access was obtained, an intravenous bolus of 5.000 U of unfractionated heparin
20 was administered, with sufficient supplements (if necessary) to maintain an activated clotting time
21 (ACT) \geq 250 seconds during interventions.

22 After baseline collection of peripheral blood samples, patients were randomized to an intravenous
23 infusion of GSH (2500 mg/25 ml of Glutathione Sodium Salt, Biomedica Foscama Group, Rome,
24 Italy) or placebo (saline solution) over 10 min before p-PCI. The two solutions appeared identical
25 in size and colour to ensure blinding. Study participants, investigators and the laboratory staff

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3 1 remained blinded until the statistical analysis was performed by an independent researcher who was
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5 2 not involved in the study.

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7 3 Patients underwent p-PCI according to standard protocols. The use of thrombus aspiration,
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9 4 glycoprotein IIb/IIIa inhibition was left to the discretion of the treating physician. Multivessel PCI
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11 5 was performed in a staged fashion (7 to 10 days from index procedure).

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14 6 All patients had drug-eluting stents implanted in treated vessels. After interventions, GSH was
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16 7 infused at the same doses at 24, 48, 72 hours elapsing time. Further blood samples were obtained at
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18 8 the end of procedure and 5 days from index procedure.

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21 9 After 60'-90', a post-procedural 12 leads- ECG for ST measurement were performed.

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24 10 Corrected TIMI frame count (cTFC) and TIMI myocardial perfusion grade (TMPG) were assessed
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26 11 after pPCI as previously described [21]. An external Core Lab processed the data (G.P and G.P:
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28 12 independent cardiologists). Digital angiograms were analyzed off-line with the use of an automated
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30 13 edge detection system (Cardiovascular Medical System, MEDIS Imaging Systems, Leiden, the
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32 14 Netherlands).

33 34 35 15 **Randomization and blinding**

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38 16 An individual not involved in the study assigned codes (using a computer-generated random
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40 17 sequence) to the study treatment with a random allocation of patients to an intravenous infusion of
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42 18 GSH (2500 mg/25 ml over 10 min) or placebo (saline solution) before p-PCI. The interventional
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44 19 cardiologists who performed p-PCI, those who analyzed digital angiograms and the laboratory
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46 20 technicians were unaware of study treatment allocation.

47 48 49 21 **Primary Endpoint**

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52 22 The primary endpoint was the change on oxidative stress markers levels after 2 hours from p-PCI in
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54 23 patients treated with GSH as compared with placebo.

55 56 24 **Secondary Endpoints**

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59 25 The secondary endpoints included the assessment of: (i) changes of oxidative stress markers levels
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26 after 5 days from the p-PCI in patients received GSH or placebo; (ii) changes in serum cTnT,

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3 1 biochemical markers of myocardial cell damage, in patients received GSH or placebo before and
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5 2 after 5 days from the procedure.

3 **Peripheral blood samples**

4 Blood samples were drawn from antecubital vein, before the start of procedure and after stent
5 deployment in all patients and then collected into tubes without anticoagulant or with 3.8% sodium
6 citrate, lithium heparin and EDTA and centrifuged at 300×g for 10 min to obtain supernatant. All
7 plasma and serum aliquots were stored at -80°C in appropriate cuvettes until assayed.

8 Markers of oxidative stress and antioxidant system (i.e. H₂O₂, H₂O₂ breakdown activity (HBA) and
9 8-iso-PGF₂α) were analyzed in serum samples collected before p-PCI, 2 hours and 5 days after p-
10 PCI. Due to the chemical properties of the oxidative stress markers, to avoid a long-time storage of
11 blood samples and guarantee the laboratory test quality the analyses were performed within 6
12 months from the collection.

13 Serum cardiac Troponin T (cTpT) was measured at admission, before the procedure, 6 and 12 hours
14 after reperfusion, and thereafter once a day up to 5 days. Serum cTpT levels were measured using
15 ELISA Kit (Elabsciences).

16 **H₂O₂ production**

17 The H₂O₂ was evaluated by a Colorimetric Detection Kit (Arbor Assays, Ann Arbor, Michigan, US)
18 and expressed as μM. Intra-assay and inter-assay coefficients of variation were 2.1% and 3.7%,
19 respectively.

20 **Determination of % HBA in peripheral serum**

21 The evaluation of the ability to detoxify H₂O₂ was assessed by the analysis of **the HBA** in serum
22 with HBA assay kit (Aurogene, Rome, Italy, code HPSA-50). The % of HBA was calculated
23 according to the following formula: % of HBA = [(Ac-As) / Ac] X 100 where Ac is the absorbance
24 of H₂O₂ 1.4 mg/ml and As is the absorbance in the presence of the serum sample.

25 **Serum Nitric Oxide (NO) bioavailability**

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3 1 A colorimetric assay kit (Cell Biolabs, San Diego, CA, US) was used to determine NO
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5 2 bioavailability by measurement of the nitric oxide metabolites nitrite and nitrate (NO_x) in the
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7 3 serum. Intra-assay and inter-assay coefficients of variation were 2.9% and 1.7% respectively.

4 **Serum 8-iso-Prostaglandin F_{2α} formation**

5 Concentration of 8-iso-PGF_{2α} in serum was measured by validated enzyme immunoassay (EIA)
6 method (DRG International, Springfield, NJ, USA). Intra-assay and inter-assay coefficients of
7 variation were 5.8% and 5.0% respectively. Values were expressed as pmol/L.

8 **Myocardial function**

9 After 120 minutes and 5 days from the intervention, left ventricular end-diastolic volume
10 (LVEDV), left ventricular end-systolic volume (LVESV) and ejection fraction (LVEF) were
11 calculated by the biplane Simpson's rule, as recommended by the American Society of
12 Echocardiography. The mean values of three measurements were used for statistical evaluation.

13 **Sample Size Calculation**

14 For the present preliminary analysis, the sample size calculation was estimated considering previous
15 data available for 8-iso-PGF_{2α} levels [22]. We hypothesized a mean difference of 20% in plasmatic
16 8-iso-PGF_{2-α} levels measured at the end of successfully reperfusion when comparing the GSH
17 group with the controls. We also assumed a SD of 50 pg/ml in each group. Based on these
18 assumptions, this study needs 25 patients for each treatment arm for a power of ≥80% with a 2-
19 sample t test at level 5%.

20 **Statistical analysis**

21 Categorical variables were reported as counts (percentage) and continuous variables as means ±
22 standard deviation (SD). We tested the independence of categorical variables by χ^2 test and the
23 normal distribution of continuous variables by Kolmogorov-Smirnov test. We used Student paired
24 and unpaired t test, repeated measure ANOVA and Pearson product-moment correlation analysis to
25 evaluate normally distributed continuous variables. Appropriate nonparametric tests (Mann-
26 Whitney U test, Wilcoxon rank test and Spearman rank correlation test) were employed for all the

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3 1 other variables. As an overall nonparametric ANOVA, the Friedman test for the analysis of
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5 2 intragroup variations was used. In cases of significance, we compared pair related samples using the
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7 3 Wilcoxon test. The intergroup analysis was performed with the nonparametric Mann–Whitney U-
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9 4 test. Only two-tailed probabilities were used for testing statistical significance. Probability values <
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11 5 0.05 were regarded as statistically significant. All calculations were made with the computer
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13 6 program STATISTICA 7 (StatSoft, Tulsa, OK, USA).
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18 19 8 **Results**

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21 9 Twenty-five patients randomly received GSH and 25 placebo. All patients completed the phases of
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23 10 the study (Figure 1). All patients had a TIMI flow grade equal to 0 or 1 requiring percutaneous
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25 11 treatment. Clinical and angiographic characteristics of patients are shown in Table 1 and 2. The
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27 12 baseline characteristics were well balanced between the two groups. In both groups, neither side
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29 13 effects during the infusion, nor adverse events during the short observation period were recorded.
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33 14 **Oxidative stress, antioxidant status and vascular function in peripheral samples.** Biochemical
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35 15 data are summarized in Table 3. Baseline H₂O₂ and 8-iso-PGF₂α levels were similar between
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37 16 treated patients and controls. After PCI, a significant reduction of H₂O₂ production and 8-iso-
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39 17 PGF₂α levels was observed in GSH group as compared to controls (**Figure 2A and 2B**). Moreover,
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41 18 a significant increase in HBA and NO bioavailability was observed (**Figure 2C and 2D**).
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45 19 At the 5 days from index procedure, a persistent significant reduction of H₂O₂ production and a
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47 20 sustained increase in HBA and NO bioavailability was observed in the GSH group as compared
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49 21 with controls (**Figure 2A-D**).
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52 22 **Serological markers of myocardial injury.** Baseline cTpT mean values were similar between
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54 23 GSH and placebo groups (176.0 ± 20.9 pg/ml vs. 165.4± 20.9 pg/ml, p=0.079). At 6 hours, no
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56 24 changes in cTpT values were found in GSH-treated patients (172.1±27.7 pg/ml vs. baseline,
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58 25 p=0.065). At 12 hours and 5 days after pPCI, GSH-treated patients showed a progressive decrease
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60 26 of cTpT levels (170.0 ± 44.7 pg/ml and 137.9 ± 23.7 pg/ml; -21±23.1%, p=0.009 vs. baseline).

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Differently, a significant increase and persistence of high values of cTpT were observed in placebo group (T6, 169.9 ± 16.3 pg/ml, T12, 183.0 ± 34.8 pg/ml and T5d, 181.9 ± 18.0 pg/ml; $+12.4 \pm 23.1\%$, $p=0.029$ vs. baseline) (**Figure 3A**). A modest correlation between percentage changes of H_2O_2 and cTpT levels from baseline to 5 days was found in treated group (**Figure 3B**).

Myocardial Reperfusion indexes. Post-procedural cTFC values did not show a statistically significant reduction between treated and control groups (20.7 ± 7.3 vs. 23.4 ± 5.1 , $p=0.156$). Interestingly, 6 patients (24%) in the placebo group and 15 (60%) patients in GSH group reached lower-risk (≤ 20 frames/s) cTFC class ($p=0.019$). After PCI, $TMPG \geq 2$ was assessed in 21 patients (84%) and 14 patients (56%) of the GSH and placebo groups, respectively ($p = 0.064$). Of note, 11 patients (44%) of the GSH group only had $TMPG=3$ ($p=0.0002$ vs. controls). Post-reperfusion cTFC values showed a significant correlation with changes of 8-iso-PGF 2α ($R=0.55$, $p=0.012$) levels from baseline.

Myocardial function. Myocardial function was not different between groups after either baseline or at discharge. There was no significant difference between groups regarding LVEF, LVEDV or LVESV at any time point (Table 4).

Discussion

This pilot study demonstrates that in the setting of STEMI reperfusion the rapid onset and prolonged antioxidant (scavenging) activity obtained by infusion of GSH before and after primary PCI reduces the oxidative stress markers. The improvement of the antioxidant status resulted in a significant decrease of cardiac troponin, marker of myocardial damage.

Data from experimental and clinical studies suggest that following reperfusion myocardial cells death largely contributes to the final infarct size.[23,24] On the other hand, the extent of damaged myocardium is the most important predictor of adverse ventricular remodeling and it is linearly dependent upon the amount of myocardial salvage by and after reperfusion. Thus, attenuation of pro-oxidant state is an important goal in cardioprotective interventions.[25]

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3 1 Noteworthy, the serum of GSH treated patients showed a greater capacity to detoxify H₂O₂
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5 2 evaluated by the HBA, an assay that measure the percentage of H₂O₂ neutralized into the
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7 3 samples.[26] We found an early and considerable increase of HBA, with positive effects on
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9 4 myocardial cell survival, assessed by cTpT.

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12 5 Current evidences demonstrate that oxidant environment promotes cardiomyocyte death in
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14 6 the first few minutes of reflow suggesting the existence of a tight window of effective cardio-
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16 7 protection.[27,28] Therefore, ROS-induced injury may continue for weeks to months as a result of
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18 8 activation of programmed cell death. Our data have shown a persistent heightened oxidative status
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20 9 along with decreased scavenging activity in untreated patients. This behavior makes the duration of
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22 10 pharmacologic interventions a central point of cardio-protection strategies. In the present study,
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24 11 GSH infusion, starting just before reperfusion with subsequent administration up to 3 days after,
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26 12 promoted early and sustained increase of serum HBA with attenuated production of H₂O₂ which
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28 13 was highly related to progressive significant reduction of serological signs of myocardial injury. In
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30 14 addition, our data show a progressive significant decrease of serum cTpT release during the 5 days
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32 15 of reperfusion in the GSH-treated patients compared with the control group resulting in a 21%
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34 16 reduction of myocardial damage. Despite that, in our population, the systolic function was not
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36 17 different between groups after reperfusion, although a trend towards reduced LVEDV was observed
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38 18 in treated patients. A possible explanation relies on the fact that inside the area at risk variable
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40 19 amount of hibernated and stunned myocardium may coexist, thus affecting the prompt recovery of
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42 20 contractility after reperfusion.[29]

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45 21 Cells have a number of mechanisms for dealing with the toxic effects of oxygen. One of the
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47 22 most important is connected with the widely distributed tripeptide thiol glutathione.[16,30] In
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49 23 particular, the glutathione redox cycle is a more efficient antioxidant protective mechanism of the
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51 24 heart, which acts by maintaining thiol groups of enzymes and other proteins in their reduced state
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53 25 thus preventing cell membrane lipid peroxidation and limiting cardiomyocytes loss.[31]
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55 26 Furthermore, in our study, a close relation between reduced myocardial reperfusion and increased
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of 8-iso-PGF 2α serum levels has been observed, suggesting that oxidative unbalance may be involved in microcirculation functional damage. As previously reported, impaired tissue-level perfusion develops within minutes of established acute revascularization of ischemic areas[32] and persists for at least 1 week.[33] In this context, there is robust evidence that ROS mediated isoprostanes production contributes importantly to the post-reperfusion microvascular impairment.[22,34] Current findings implement this observation by demonstrating a sustained production of isoprostanes up to 5 days after reperfusion thereby suggesting their contributory role in the pathogenesis and persistence of microvascular dysfunction that may affect myocardial cell survival. The infusion of GSH before and 24, 48, 72 hours after pPCI reduced isoprostanes serum levels and their reduction was linked to improvement of myocardial reperfusion indexes. Moreover, the increase in extracellular peroxide oxidants may reduce bioavailability of nitric oxide that is thought to contribute to promoting platelet hyperactivity and vasoconstriction.[13] In our study, GSH supplementation seems to have a role in preserving NO bioavailability and its vasodilator capacity at microcirculatory level.

The Strengths and Limitations of the Study

The positive effects on reperfusion indexes and on biochemical signs of myocardial necrosis suggest the value of prophylactic and prolonged GSH administration in preventing reperfusion injury. Thus, in patients undergoing pPCI the infusion of a powerful antioxidant scavenger, such as GSH, may be useful to improve microcirculatory perfusion in order to further blunt the injury of myocardial cells.

Some limitations deserve to be discussed.

The small sample size of the study and the lack of morfologic assessment of both infarct size and microvascular obstruction extent between the two groups, actually, limit the clinical application of these findings. Within a defined area at risk, the manifestations of ischemia-reperfusion vascular injury go from reversible functional impairments to irreversible structural damage and contribute to final amount of infarct myocardium. In absence of morphologic imaging, the qualitative evaluation

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3 1 of GSH-induced improvement of myocardial reperfusion indexes, as assessed in our study, might
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5 2 only represent the effect of a preserved microcirculatory responsiveness to vasoactive substances
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8 3 (i.e. NO) but unable to limit the expansion of myocardial cell damage. Indeed, other mechanisms,
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10 4 such as interstitial edema and inflammatory reaction, which induce a sustained impairment of
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12 5 microvascular perfusion, may primarily act to increase the amount of irreversible injured
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14 6 myocardium thus promoting adverse ventricular remodeling.

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17 7 In conclusion, in this pilot study, we have shown that a short-term prophylactic GSH
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19 8 infusion mitigates the negative effects of the excessive and persistent H₂O₂ formation on myocardial
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21 9 cells. The findings of the present study require to be confirmed through an adequately powered
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24 10 STEMI population. A larger trial with a prolonged follow-up for evaluation of clinical endpoints is
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26 11 needed to confirm the role of GSH administration as cardioprotective therapy.

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5 3 **Figure Legend**
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10 6 **Figure 1. CONSORT flowchart**
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14 8 **Figure 2.** H₂O₂ production (A), 8-iso-PGF₂α formation (B), hydrogen peroxide breakdown activity
15 9 (HBA) (C) and NO bioavailability (D) at baseline, after 2 hours (T2h) and at the 5 days (T5d) from
16
17 10 the PCI in patients received GSH (n=25, dashed line) or placebo (n=25, continuous line).
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21 11 Data are expressed as mean±SEM (***p <0.001, **p <0.01, *p <0.05).
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26 13 **Figure 3.** cT_pT levels (A) at baseline, after 6 hours (T6h), 12 hours (T12h) and at the 5 days (T5d)
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28 14 from the PCI in patients received GSH (n=25, dashed line) or placebo (n=25, continuous line). Data
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30 15 are expressed as mean±SEM (***p<0.0001 vs. T0, *p<0.05 vs. T0, \$p<0.05 between groups).
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33 16 Linear correlation between % Δ cT_pT and % Δ H₂O₂ in GSH treated group (B).
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10 4 **Contributors**

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12 5 GTa, EM led on the conception, design and writing of the study with substantial contributions to the
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14 6 design, writing, critical review of intellectual content; GTr, AA, AP, NV, RM, enrollment of
15
16 7 patients; RC, VC, CN, provided laboratory analyses; VR, SB, provided further essential statistical
17
18 8 advice and expertise on the study protocol; FB, LL, MP, CG, providing expert clinical support;
19
20 9 AG, MD, made substantial contributions to the trial design and management.
21
22

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24 10 **Ethics and dissemination**

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26 11 Italian Medicines Agency (AIFA) authorization and single Ethic Committee approval has been
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28 12 obtained for all the centers participating the study. Dissemination of results will be via peer-
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30 13 reviewed research publications both online and in print, conference presentations, posters, patient
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32 14 forums and Trust bulletins.
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35 15 **Data Availability Statement**

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37 16 The data set is available on request from the corresponding author.
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39

40 17 **Conflicts of Interest**

41
42 18 Authors declare that there are not conflicts of interest.
43

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45

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48 21 the external assessment of angiographic data (Core Lab)
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4 obstruction and tissue injury after acute myocardial infarction. *Circulation* 1998;98:1006-1014.
- 5 34 Yaqin X, Huo Y, Toufektsian MC, et al. Activated platelets contribute importantly to myocardial
6 reperfusion injury. *Am J Physiol Heart Circ Physiol* 2006;290:H692-H699.

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Table 1. Clinical characteristics of the study population

Variables	GSH group (n=25)	Placebo group (n=25)	P value
Age (y, mean±SD)	66 ± 10.7	66.9 ± 9.1	0.74
Male , n (%)	15 (60)	13 (52)	0.98
Body-mass index § (mean±SD)	26.9 ± 3.9	20 ± 3.8	0.38
Killip class ≥ 3 , n (%)	2 (8)	0 (0)	0.47
Diabetes Mellitus , n (%)	5 (20)	5 (20)	1
Hypertension , n (%)	14 (56)	17 (68)	0.56
Dyslipidemia , n (%)	11 (44)	13 (52)	0.77
Statin use , n (%)	8 (32)	8 (32)	1
Smokers , n (%)	17 (68)	13 (52)	0.38

§ The body-mass index is the weight in kilograms divided by the square of the height in meters.

Table 2. Angiographic parameters

Variables	GSH group (n=25)	Placebo group (n=25)	P value
Ischemia time# (min; mean \pm SD)	286 \pm 88	270 \pm 96	0.85
Thrombus Burden \geq 3, n (%)	12 (48)	11 (44)	0.77
Thrombus aspiration, n (%)	13 (52)	12 (48)	0.87
GP IIb/IIIa inhibitors, n (%)	2 (8)	3 (12)	0.63
MVD, n (%)	13 (52)	11 (44)	0.77
2 vessels,	8 (32)	5 (20)	0.89
3 vessels,	5 (20)	6 (24)	
Staged PCI, n (%)	9 (36)	5 (20)	
IRA:			
LAD, n (%)	10 (40)	9 (36)	0.77
LCx, n (%)	5 (20)	6 (24)	0.73
RCA, n (%)	10 (40)	10 (40)	1

PCI = percutaneous coronary intervention; **IRA** = infarct related coronary artery; **LAD** = left anterior descending coronary artery; **LCx** = left circumflex coronary artery; **RCA** = Right coronary artery. #ischemia time was defined as the timing between symptom onset and balloon inflation.

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Table 3. Biochemical data

	Baseline			Reperfusion 2h			Follow-up (5 days)		
Variable	GSH	Placebo	p	GSH	Placebo	p	GSH	Placebo	p
H₂O₂ μM, mean±SD	40.6±8.4	43.6±11.6	0.305	28.4±12	42.8±14.1	0.0003	24±7	39.5±17.3	0.0001
Δ				-12.1±15.2	-0.7±17.9	0.03	-16.6±11.0	-4.1±20.14	0.009
8-iso-PGF2α pmol/L, mean±SD	214.6±81.1	211.9±92.1	0.91	163.6±44.7	217.6±51.6	0.0003	159.9±34.2	213.1±50.9	0.0001
Δ				-50.9±92.9	-3.3±1.29	0.02	-54.6±62.1	-1.2±115.7	0.02
HBA %, mean±SD	43.6±7.4	43.4±11.9	0.94	57.9±8.6	43.9±8.7	0.0001	62.9±10.5	45.2±13.0	0.0001
Δ				+14.9±5.5	+0.4±14.9	0.0004	+19.4±10.2	+1.8±17.1	0.0001
NO μM, mean±SD	16.3±5.7	16.5±4.7	0.89	27.7±7.2	22.4±10	0.0356	35.5±8.1	23.5±15.5	0.0013
Δ				+11.4±6.8	+5.8±10.5	0.05	+19.2±9.7	+7.0±14.7	0.002

GSH = reduced Glutathione; H₂O₂ = Hydrogen Peroxide; 8-iso-PGF2α = 8-iso-Prostaglandin-F2α;

HBA = H₂O₂ break-down activity

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Table 4. Left Ventricular echocardiographic parameters at baseline and at follow-up

Echo parameters	Placebo (n= 25)	GSH (n=25)	P-value
Baseline			
LVEDV (mL/m²)	121.3 ± 17.2	124.4 ± 22.3	0.44
LVESV (mL/m²)	65.4 ± 11.3	66.3± 13.2	0.91
LVEF (%)	47.5 ± 4.9	46.9 ± 4.8	0.42
Follow-up			
LVEDV (mL/m²)	118.1 ± 17.8	113.2 ± 14.1	0.42
LVESV (mL/m²)	60.9 ± 10.7	58.8± 12.5	0.91
LVEF (%)	49.1 ± 3.2	49.8 ± 3.7	0.42

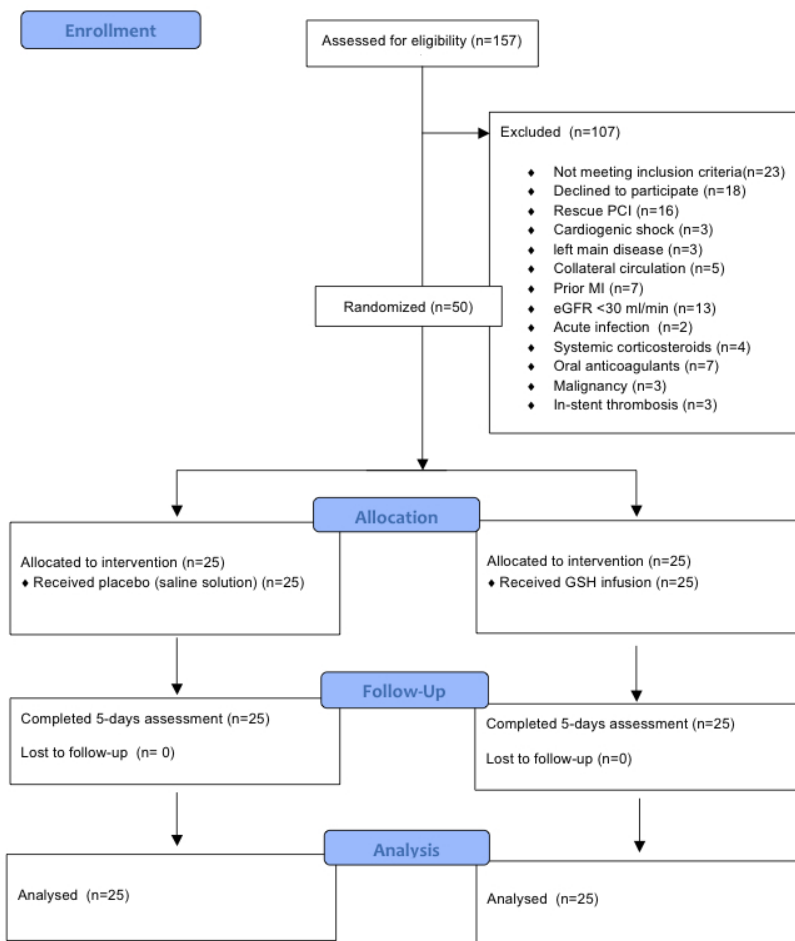
LVEDV = left ventricular end-diastolic volume; **LVEDSV** = left ventricular end-systolic volume; **LVEF** = left ventricular ejection fraction-diastolic volume

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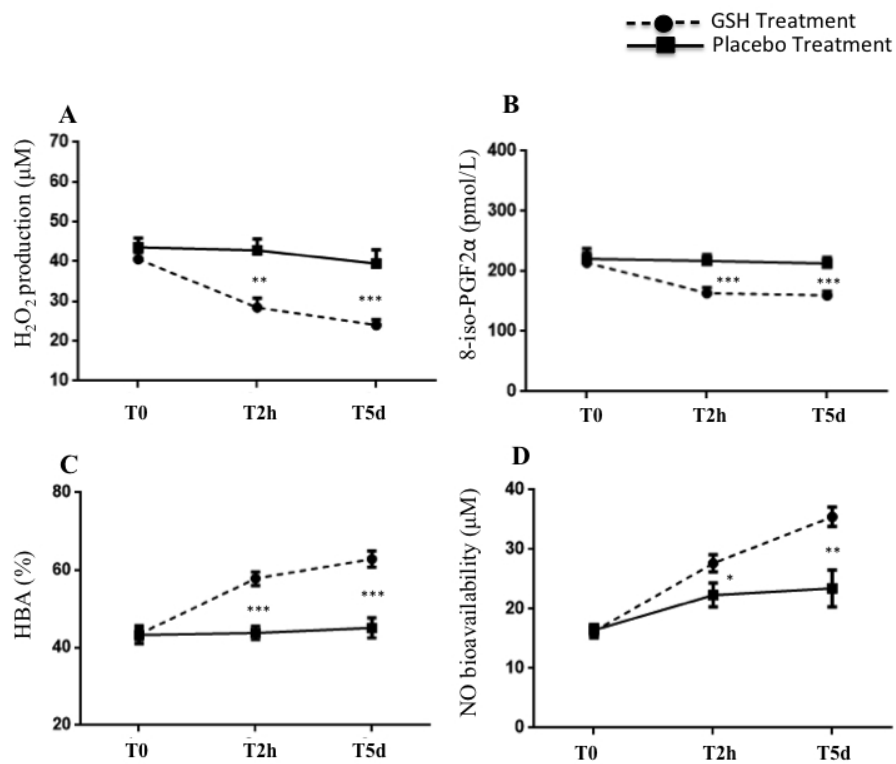
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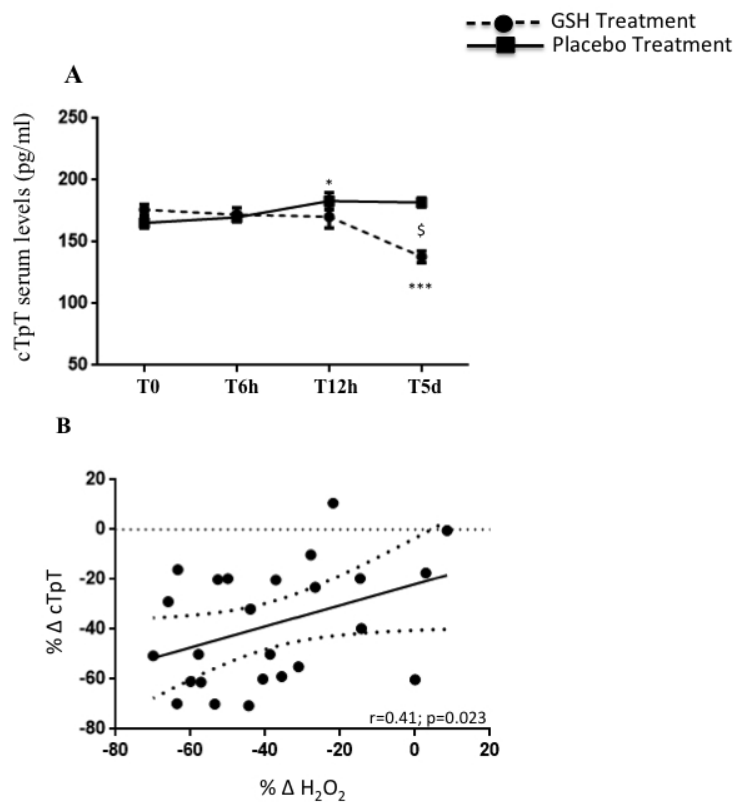
CONSORT flowchart



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3 **EudraCT Number: 2014-004486-25**

4 **Sponsor's Protocol Code Number: GSH2014**

5 National Competent Authority: Italy - Italian Medicines Agency

6 Clinical Trial Type: EEA CTA

7 Trial Status: submitted data of the pilot study. The trial is ongoing.

8 Date on which this record was first entered in the EudraCT database: 2014-12-04

9 Link: <https://www.clinicaltrialsregister.eu/ctr-search/trial/2014-004486-25/IT/>

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13 **A. Protocol Information**

14 Member State Concerned: Italy - Italian Medicines Agency

15 EudraCT number: 2014-004486-25

16 Full title of the trial: Prevention of the reperfusion myocardial damage in patients with acute
17 myocardial infarct (STEMI) submitted to primary PCI through infusion of intravenous glutathione.

18 Sponsor's protocol code number: GSH2014

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21 **B. Sponsor Information**

22 Sponsor 1: University Hospital "Policlinico Umberto I"

23 Name of organization providing support: University Hospital "Policlinico Umberto I", Rome, Italy.

24 Functional name of contact point: Enrico Mangieri, University Hospital "Policlinico Umberto I".

25 Viale del Policlinico, 155 – Rome, Post code: 00161, Italy

26 E-mail: enrico.mangieri@uniroma1.it

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29 **D. IMP Identification**

30 *IMP to be used in the trial has a marketing authorisation: Yes*

31 *Trade name: TAD*

32 Name of the Marketing Authorisation holder: Biomedica Foscama Group S.p.A.

33 Country which granted the Marketing Authorisation: Italy

34 Pharmaceutical form: Powder and solvent for solution for infusion

35 Routes of administration for this IMP: Intravenous use

36 *Information on Placebo*

37 Pharmaceutical form of the placebo: saline solution

38 Route of administration of the placebo: Intravenous use

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42 **E. General Information on the Trial**

43 Medical condition or disease under investigation

44 Medical condition(s) being investigated: ST-Segment Elevation Myocardial Infarction (STEMI).

45 Medical condition in easily understood language: acute myocardial infarct

46 Therapeutic area: Diseases [C] - Cardiovascular Diseases [C14]

47 Objective of the trial

48 Main objective of the trial: To verify if the intravenous infusion of "Glutathione Sodium Salt" it is
49 able to reduce the level of oxidative state in the area of myocardial infarction.

50 Secondary objectives of the trial: To verify if the intravenous infusion of "Glutathione Sodium Salt"
51 during the procedures of primary PCI it is able to limit the extension of the ischemic area, to
52 reduce the incidence of the no-reflow, to improve the degree of myocardial blush and to decrease
53 the indexes of suffering post-procedural ischemia (ST elevation; release of myocardial necrosis
54 markers).

55 Principal inclusion criteria: STEMI patients submitted to p-PCI up to 12 hours.

56 Age ≥ 18 years. Women and Men. Signed informed consent

Principal exclusion criteria:

Patients with cardiac arrest, ventricular fibrillation, cardiogenic shock, stent thrombosis, previous acute myocardial infarction, or angina within 48 hours before infarction were not included in the study. Patients with evidence of coronary collaterals (2-3 Rentrop) to the region at risk on initial coronary angiography (at the time of admission) will be excluded. Moreover, patients with EF $\leq 30\%$, impaired renal function (creatinine > 3.0 mg/dl), recipient of heart transplant, a life expectancy less than 12 months, has known allergies to aspirin, clopidogrel bisulfate, heparin, contrast media or stainless steel that cannot be managed medically were excluded. Patient needs therapy with warfarin or currently participating in an investigational drug or another device study were not considered enrolling.

End points

Primary end point(s): The primary endpoint will consist in the assessment of the effects of the infusion of "Glutathione Sodium Salt" on the reduction of the oxidative markers and inflammation after PCI.

Timepoint(s) of clinical evaluation of this end point: before, 2 hour and 5 days from the p-PCI

Secondary end point(s): The secondary endpoint will include: (1) the assessment of the variations of the corrected TIMI frame count (cTFC) and the TIMI Myocardial Perfusion Grade (TMPG) after p-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 occurrence of a new revascularization, up to 6 months after the procedure.

Medical Doctors don't have the knowledge both about the possible infusion of the Glutathione Sodium Salt, in the examined patient, then others clinical data.

Moreover, serological levels of Troponin and creatinine will be measured before the p-PCI and after the procedure (2, 6, 12 and 24 hours).

Besides, through 2D Echocardiography with Simpson's biplane method the FE will be calculate at admission and after hospital discharge.

If clinical-instrumental signs of ischemia will rise up, the patient will be submitted to a new angiography.

Definition of the end of the trial and justification where it is not the last visit of the last subject undergoing the trial: LVLS or telephonic contact

Population of Trial Subjects

Trial has subjects under 18: No

Adults (18-64 years): Yes

Number of subjects for this age range: 30

Elderly (≥ 65 years): Yes

Number of subjects for this age range: 60

Female: Yes

Male: Yes

Patients: Yes

Specific vulnerable populations: Yes

Women of childbearing potential not using contraception: Yes

Women of child-bearing potential using contraception: Yes

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Pregnant women: No

Nursing women: No

Emergency situation: No

Subjects incapable of giving consent personally: No

Planned number of subjects to be included: 90

F. Investigator Networks to be involved in the Trial

N. Review by the Competent Authority or Ethics Committee in the country concerned

N. Competent Authority Decision: Authorised

N. Date of Competent Authority Decision: 2015-01-13

N. Ethics Committee Opinion of the trial application: Favourable

N. Date of Ethics Committee Opinion: 2015-02-12

N. Centers involved in the study: Department of Heart and Great Vessel "A. Reale", Sapienza University of Rome (coordinator centre) - "Santa Maria" Terni Hospital - "San Giovanni Evangelista" Tivoli Hospital, all in Italy.

P. End of Trial Status: analyzed as pilot study the first 50 enrolled patients. Ongoing.



CONSORT 2010 checklist of information to include when reporting a randomised trial*

Section/Topic	Item No	Checklist item	Reported on page No
Title and abstract			
	1a	Identification as a randomised trial in the title	1
	1b	Structured summary of trial design, methods, results, and conclusions (for specific guidance see CONSORT for abstracts)	2
Introduction			
Background and objectives	2a	Scientific background and explanation of rationale	4
	2b	Specific objectives or hypotheses	5
Methods			
Trial design	3a	Description of trial design (such as parallel, factorial) including allocation ratio	6
	3b	Important changes to methods after trial commencement (such as eligibility criteria), with reasons	NA
Participants	4a	Eligibility criteria for participants	6
	4b	Settings and locations where the data were collected	7
Interventions	5	The interventions for each group with sufficient details to allow replication, including how and when they were actually administered	7
Outcomes	6a	Completely defined pre-specified primary and secondary outcome measures, including how and when they were assessed	7-8
	6b	Any changes to trial outcomes after the trial commenced, with reasons	NA
Sample size	7a	How sample size was determined	9
	7b	When applicable, explanation of any interim analyses and stopping guidelines	9
Randomisation:			
Sequence generation	8a	Method used to generate the random allocation sequence	7
	8b	Type of randomisation; details of any restriction (such as blocking and block size)	7
Allocation concealment mechanism	9	Mechanism used to implement the random allocation sequence (such as sequentially numbered containers), describing any steps taken to conceal the sequence until interventions were assigned	7
Implementation	10	Who generated the random allocation sequence, who enrolled participants, and who assigned participants to interventions	7
Blinding	11a	If done, who was blinded after assignment to interventions (for example, participants, care providers, those	7

1		assessing outcomes) and how	
2	11b	If relevant, description of the similarity of interventions	NA
3	Statistical methods	12a	Statistical methods used to compare groups for primary and secondary outcomes
4		12b	Methods for additional analyses, such as subgroup analyses and adjusted analyses
5			NA
6	Results		
7	Participant flow (a	13a	For each group, the numbers of participants who were randomly assigned, received intended treatment, and
8	diagram is strongly		were analysed for the primary outcome
9	recommended)	13b	For each group, losses and exclusions after randomisation, together with reasons
10	Recruitment	14a	Dates defining the periods of recruitment and follow-up
11		14b	Why the trial ended or was stopped
12			NA
13	Baseline data	15	A table showing baseline demographic and clinical characteristics for each group
14	Numbers analysed	16	For each group, number of participants (denominator) included in each analysis and whether the analysis was
15			by original assigned groups
16			10
17	Outcomes and	17a	For each primary and secondary outcome, results for each group, and the estimated effect size and its
18	estimation		precision (such as 95% confidence interval)
19		17b	For binary outcomes, presentation of both absolute and relative effect sizes is recommended
20			NA
21	Ancillary analyses	18	Results of any other analyses performed, including subgroup analyses and adjusted analyses, distinguishing
22			pre-specified from exploratory
23			NA
24	Harms	19	All important harms or unintended effects in each group (for specific guidance see CONSORT for harms)
25			10
26	Discussion		
27	Limitations	20	Trial limitations, addressing sources of potential bias, imprecision, and, if relevant, multiplicity of analyses
28	Generalisability	21	Generalisability (external validity, applicability) of the trial findings
29	Interpretation	22	Interpretation consistent with results, balancing benefits and harms, and considering other relevant evidence
30			14
31	Other information		
32	Registration	23	Registration number and name of trial registry
33			5
34	Protocol	24	Where the full trial protocol can be accessed, if available
35			Supplementary
36	Funding	25	Sources of funding and other support (such as supply of drugs), role of funders
37			file
38			16

38 *We strongly recommend reading this statement in conjunction with the CONSORT 2010 Explanation and Elaboration for important clarifications on all the items. If relevant, we also
 39 recommend reading CONSORT extensions for cluster randomised trials, non-inferiority and equivalence trials, non-pharmacological treatments, herbal interventions, and pragmatic trials.
 40 Additional extensions are forthcoming: for those and for up to date references relevant to this checklist, see www.consort-statement.org.
 41
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BMJ Open

Glutathione infusion before primary percutaneous coronary intervention: a randomized controlled pilot study.

Journal:	<i>BMJ Open</i>
Manuscript ID	bmjopen-2018-025884.R2
Article Type:	Research
Date Submitted by the Author:	12-Apr-2019
Complete List of Authors:	Tanzilli, Gaetano; University of Rome La Sapienza, Department of Heart and Great Vessels, Truscelli, Giovanni; Sapienza University of Rome Arrivi, Alessio; Department of Cardiology Carnevale, Roberto; Sapienza University, I Clinica Medica Placanica, Attilio; Department of Cardiology Viceconte, Nicola; Università degli studi di Roma "La Sapienza", Dipartimento Cuore e Grossi Vasi Raparelli, Valeria ; Sapienza University of Rome, I Clinica Medica, Department of Internal Medicine and Medical Specialties Mele, Rita; Sapienza University Cammisotto, Vittoria; Sapienza University, Department of Internal Medicine and Medical Specialties Nocella, Cristina; IRCCS NeuroMed Barillà, Francesco; Sapienza University , Department of the Heart and Great Vessels Lucisano, Luigi; Department of Cardiology Pennacchi, Mauro; Department of Cardiology Granatelli, Antonino; Department of Cardiology Dominici, Marcello; Department of Cardiology Basili, Stefania; Sapienza University of Rome, I Clinica Medica Gaudio, Carlo; University of Rome La Sapienza Mangieri, Enrico; Policlinico Umberto I, Sapienza University, Cuore e Grossi Vasi
Primary Subject Heading:	Cardiovascular medicine
Secondary Subject Heading:	Pharmacology and therapeutics
Keywords:	Glutathione, Reperfusion Injury, STEMI, hydrogen peroxide

SCHOLARONE™
Manuscripts

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3 1 **Glutathione infusion before primary percutaneous coronary intervention: a randomized**
4
5 2 **controlled pilot study.**

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7 3 Gaetano Tanzilli¹, Giovanni Truscelli¹, Alessio Arrivi², Roberto Carnevale^{3,4}, Attilio Placanica⁵,
8 4 Nicola Viceconte¹, Valeria Raparelli⁶, Rita Mele⁷, Vittoria Cammisotto⁸, Cristina Nocella⁸,
9
10 5 Francesco Barillà¹, Luigi Lucisano⁵, Mauro Pennacchi⁵, Antonino Granatelli⁵, Marcello Dominici²,
11
12 6 Stefania Basili⁶, Carlo Gaudio¹ and Enrico Mangieri¹
13
14
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18
19 8 ¹ Department of the Heart and Great Vessels, Sapienza University of Rome, Rome, Italy

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21 9 ² Department of Cardiology, "Santa Maria" Hospital, Terni, Italy

22
23 10 ³ Department of Medical-Surgical Sciences and Biotechnologies, Sapienza University, Latina, Italy

24
25 11 ⁴ Mediterranea Cardiocentro, Napoli

26
27 12 ⁵ Department of Cardiology, "San Giovanni Evangelista" Hospital, Tivoli, Italy

28
29 13 ⁶ Department of Experimental Medicine, Sapienza University of Rome, Rome, Italy

30
31 14 ⁷ Department of Surgical Sciences, Sapienza University, Rome, Italy

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33 15 ⁸ Department of Internal Medicine and Medical Specialties, Sapienza University, Rome, Italy
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40 17 **Corresponding Author:**

41
42 18 Gaetano Tanzilli, MD, Department of Heart and Great Vessels "Attilio Reale", Sapienza University
43 19 of Rome, Viale del Policlinico 155, 00161 Rome, Italy; +39 06 49973240

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1 **Abstract**

2 **Objective-** In the setting of reperfused ST-elevation myocardial infarction (STEMI), increased
3 production of reactive oxygen species (ROS) contributes to reperfusion injury. Among ROS,
4 hydrogen peroxide (H₂O₂) showed toxic effects on human cardiomyocytes and may induce
5 microcirculatory impairment. Glutathione (GSH) is a water-soluble tripeptide with a potent oxidant
6 scavenging activity. We hypothesized that the infusion of GSH before acute reoxygenation might
7 counteract the deleterious effects of increased H₂O₂ generation on myocardium.

8 **Methods-** Fifty consecutive STEMI patients scheduled to undergo primary angioplasty were
9 randomly assigned, before intervention, to receive an infusion of GSH (2500 mg/25ml over 10 min)
10 followed by drug administration at the same doses at 24, 48, 72 hours elapsing time or placebo.
11 Peripheral blood samples were obtained before and at the end of procedure as well as after 5 days.
12 H₂O₂ production, 8-iso-PGF₂ α formation, H₂O₂ breakdown activity (HBA) and nitric oxide (NO)
13 bioavailability were determined. Serum cardiac-Troponin T (cTpT) was measured at admission and
14 up to 5 days.

15 **Results-** Following acute reperfusion, a significant reduction of H₂O₂ production (p=0.0015) and 8-
16 iso-PGF₂ α levels (p=0.0003) as well as a significant increase in HBA (p<0.0001) and NO
17 bioavailability (p=0.035) was found in the GSH group as compared with placebo. In treated
18 patients, attenuated production of H₂O₂ persisted up to 5 days from the index procedure (p=0.009)
19 and these changes were linked to those of cTpT levels (r=0.41, p= 0.023).

20 **Conclusion** The prophylactic and prolonged infusion of GSH seems determined a rapid onset and
21 persistent blunting of H₂O₂ generation improving myocardial cell survival. Nevertheless, a larger
22 trial, adequately powered for evaluation of clinical endpoints, is ongoing to confirm the current
23 finding.

24
25 **Key words:** Glutathione; STEMI; Reperfusion Injury; Reactive Oxygen Species; hydrogen
26 peroxide, Percutaneous Coronary Interventions.

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3 1 **Article summary**
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5 2 **Strengths and limitations of this study**
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- 7
8 3 1. In patients who suffer from STEMI, acute reoxygenation of ischemic myocardium can
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10 4 induce additional myocardial cell injury mainly driven by heightened oxidative status.
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12 5 2. Reactive oxygen species (ROS) generation further contributes to damage myocardium by
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14 6 limiting bioavailability of nitric oxide at microcirculatory level.
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17 7 3. This pilot study demonstrates that in the setting of STEMI reperfusion the rapid onset and
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19 8 prolonged antioxidant (scavenging) activity obtained by infusion of glutathione (GSH)
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21 9 protects the myocardium.
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24 10 4. This study is limited by the lack of clinical end points and the small sample size. Moreover,
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26 11 qualitative assessment of GSH-induced improvement of myocardial reperfusion indexes,
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28 12 might only represent the effect of a preserved microcirculatory responsiveness to vasoactive
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30 13 substances (i.e. NO) but unable to limit the expansion of myocardial cell damage.
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1 Introduction

2 It is well known that reactive oxygen species (ROS) are produced at an accelerated rate in tissues
3 subjected to reperfusion and that the accumulation of ROS contributes to reperfusion injury during
4 reintroduction of molecular oxygen to the ischemic environment.[1,2]

5 In the setting of ST-elevation myocardial infarction (STEMI), ROS-induced myocardial cell death
6 occurs in the first few minutes of acute reoxygenation[3] and may continue for weeks to months by
7 activation of apoptosis and autophagy processes.[4,5] ROS generation also contributes to structural
8 capillary damage and endothelial dysfunction, which hinder the achievement of an optimal
9 perfusion grade at microcirculatory level.[6,7] Over the time, this may result in adverse left
10 ventricular (LV) remodeling and worse LV function.[8-10]

11 Among ROS, hydrogen peroxide (H_2O_2) is produced by many enzymes including for example
12 xanthine oxidase, lipoxygenase and, in particular, NADPH oxidase.[11] H_2O_2 shows an important
13 role in ischemia/reperfusion damage. In particular, the exposure of cultured human cardiomyocytes
14 to H_2O_2 has determined rapid onset and progressive oxidative cell death.[12] Moreover, H_2O_2
15 influences platelet activation and promotes vascular dysfunction through thromboxane A_2 and
16 isoprostanes formation, which are vasoconstrictors and powerful aggregating molecules derived
17 from lipid peroxidation of esterified unsaturated fatty acids.[13-15]

18 Human possesses numerous enzymatic and non-enzymatic antioxidant systems. Among enzymatic
19 system, glutathione peroxidase (GPx) plays an important role to prevent potentially deleterious
20 effects of H_2O_2 . [16] Thus, the reduced plasma level of glutathione (GSH), a water-soluble tripeptide
21 with a potent oxidant scavenging activity and fundamental substrate for GPx activity, could have a
22 key role in promoting myocardial and endothelial cell damage.[17] In fact, a decrease in myocardial
23 GSH content has been observed during ischemia and reperfusion of the ischemic myocardium.[18]
24 Despite robust evidences regarding the role of ROS in reperfusion injury, currently, in clinical
25 practice, there are no treatments aimed at preventing ROS generation.

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1 Preclinical study of ischemia/reperfusion demonstrated that timely application of GSH provides
2 better cardio-protection at higher doses [19]. Our hypothesis is that the use of GSH might
3 counteract deleterious effect of augmented oxidant activity during reperfusion of STEMI [20].
4 Currently, a glutathione solution is available for intravenous usage to reduce side effects of
5 chemotherapy treatment for cancer with a tolerable safety profile, however it has never been tested
6 in the setting of patients with STEMI.
7 Therefore, we performed a pilot study to explore whether a short-term intravenous GSH
8 administration, just before and after a primary percutaneous coronary intervention (p-PCI) in
9 STEMI patients, was able to reduce oxidative stress and antioxidant status markers, resulting in a
10 reduction of the myocardial damage.

11 **Methods**

12 **Study Design**

13 GSH2014 is a multicenter, no profit, randomized, double-blind, prospective, placebo-controlled
14 trial. The Department of Heart and Great Vessel "A. Reale", Sapienza University of Rome, Italy
15 was the coordinator center and designed the protocol (see Supplementary file). Two other centers,
16 "Santa Maria" Terni Hospital and "San Giovanni Evangelista" Tivoli Hospital, both in Italy, were
17 involved in the study as recruiting site.

18 The study has been planned according to principles of the declaration of Helsinki. Ethic Committee
19 of the coordinator centre and Italian Medicines Agency (AIFA) (Date of Competent Authority
20 Decision: 2015-01-13) authorized the study. Written informed consent was obtained from all
21 patients enrolled. (<https://www.clinicaltrialsregister.eu/ctr-search/trial/2014-004486-25/IT#N>)

22 **Patient and Public Involvement**

23 Patients and or public were not involved in the different stages of the study (including the design
24 and the recruitment phase). However, we intend to disseminate the main results to trial participants
25 and will seek patient and public involvement in the development of an appropriate method of
26 dissemination.

1 **Study population and protocol**

2 Between March and August 2017, 157 consecutive STEMI patients, age >18 years, both sexes,
3 referred to the three enrolling centers for primary percutaneous coronary intervention (p-PCI) were
4 screened to enter in the study. Inclusion criteria were: typical chest pain lasting more than 30 min
5 with pain onset <12 h, ST segment elevation >0.2 mV in at least two contiguous leads in the initial
6 ECG, successful p-PCI (residual coronary stenosis <20%) and blood sampling for biochemical
7 determinations collected prior to p-PCI.

8 Exclusion criteria were: symptoms duration > 12 h (n=15), rescue PCI (n=16), cardiogenic shock
9 (n=3), left main disease (n=3), evidence of coronary collateral vessels (Rentrop score of 2 or 3 for
10 the area at risk) (n=5), prior myocardial infarction (n=7), estimated glomerular filtration rate less
11 than 30 ml/min (n=13), acute infection (n=2), treatment with systemic corticosteroids (n=4) or oral
12 anticoagulants (n=7), malignancy (n=3), in-stent thrombosis (n=3), lack of consent to participate
13 (n=18). Additionally, 8 patients were ineligible because no blood samples were collected before the
14 start of procedure. Finally, a total of 50 patients were enrolled (see Figure 1- CONSORT diagram).

15 The present analysis reported the results of the interim analysis (pre-planned in the protocol) on the
16 acute effect of GSH infusion on markers of oxidative stress.

17 After percutaneous access was obtained, an intravenous bolus of 5.000 U of unfractionated heparin
18 was administered, with sufficient supplements (if necessary) to maintain an activated clotting time
19 (ACT) \geq 250 seconds during interventions.

20 After baseline collection of peripheral blood samples, patients were randomized to an intravenous
21 infusion of GSH (2500 mg/25 ml of Glutathione Sodium Salt, Biomedica Foscama Group, Rome,
22 Italy) or placebo (saline solution) over 10 min before p-PCI. The two solutions appeared identical
23 in size and colour to ensure blinding. Study participants, investigators and the laboratory staff
24 remained blinded until the statistical analysis was performed by an independent researcher who was
25 not involved in the study.

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3 1 Patients underwent p-PCI according to standard protocols. The use of thrombus aspiration,
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5 2 glycoprotein IIb/IIIa inhibition was left to the discretion of the treating physician. Multivessel PCI
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8 3 was performed in a staged fashion (7 to 10 days from index procedure).

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10 4 All patients had drug-eluting stents implanted in treated vessels. After interventions, GSH was
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12 5 infused at the same doses at 24, 48, 72 hours elapsing time. Further blood samples were obtained at
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14 6 the end of procedure and 5 days from index procedure.

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17 7 After 60'-90', a post-procedural 12 leads- ECG for ST measurement were performed.

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19 8 Corrected TIMI frame count (cTFC) and TIMI myocardial perfusion grade (TMPG) were assessed
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21 9 after pPCI as previously described [21]. An external Core Lab processed the data (G.P and G.P:
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23 10 independent cardiologists). Digital angiograms were analyzed off-line with the use of an automated
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26 11 edge detection system (Cardiovascular Medical System, MEDIS Imaging Systems, Leiden, the
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28 12 Netherlands).

30 13 **Randomization and blinding**

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33 14 An individual not involved in the study assigned codes (using a computer-generated random
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35 15 sequence) to the study treatment with a random allocation of patients to an intravenous infusion of
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37 16 GSH (2500 mg/25 ml over 10 min) or placebo (saline solution) before p-PCI. The interventional
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39 17 cardiologists who performed p-PCI, those who analyzed digital angiograms and the laboratory
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42 18 technicians were unaware of study treatment allocation.

43 44 19 **Primary Endpoint**

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47 20 The primary endpoint was the change on oxidative stress markers levels after 2 hours from p-PCI in
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49 21 patients treated with GSH as compared with placebo.

50 51 22 **Secondary Endpoints**

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53 23 The secondary endpoints included the assessment of: (i) changes of oxidative stress markers levels
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55 24 after 5 days from the p-PCI in patients received GSH or placebo; (ii) changes in serum cTnT,
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58 25 biochemical markers of myocardial cell damage, in patients received GSH or placebo before and
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60 26 after 5 days from the procedure.

1 **Peripheral blood samples**

2 Blood samples were drawn from antecubital vein, before the start of procedure and after stent
3 deployment in all patients and then collected into tubes without anticoagulant or with 3.8% sodium
4 citrate, lithium heparin and EDTA and centrifuged at $300\times g$ for 10 min to obtain supernatant. All
5 plasma and serum aliquots were stored at -80°C in appropriate cuvettes until assayed.

6 Markers of oxidative stress and antioxidant system (i.e. H_2O_2 , H_2O_2 breakdown activity (HBA) and
7 8-iso-PGF 2α) were analyzed in serum samples collected before p-PCI, 2 hours and 5 days after p-
8 PCI. Due to the chemical properties of the oxidative stress markers, to avoid a long-time storage of
9 blood samples and guarantee the laboratory test quality the analyses were performed within 6
10 months from the collection.

11 Serum cardiac Troponin T (cTpT) was measured at admission, before the procedure, 6 and 12 hours
12 after reperfusion, and thereafter once a day up to 5 days. Serum cTpT levels were measured using
13 ELISA Kit (Elabsciences).

14 **H_2O_2 production**

15 The H_2O_2 was evaluated by a Colorimetric Detection Kit (Arbor Assays, Ann Arbor, Michigan, US)
16 and expressed as μM . Intra-assay and inter-assay coefficients of variation were 2.1% and 3.7%,
17 respectively.

18 **Determination of % HBA in peripheral serum**

19 The evaluation of the ability to detoxify H_2O_2 was assessed by the analysis of **the HBA** in serum
20 with HBA assay kit (Aurogene, Rome, Italy, code HPSA-50). The % of HBA was calculated
21 according to the following formula: % of HBA = $[(\text{Ac}-\text{As}) / \text{Ac}] \times 100$ where Ac is the absorbance
22 of H_2O_2 1.4 mg/ml and As is the absorbance in the presence of the serum sample.

23 **Serum Nitric Oxide (NO) bioavailability**

24 A colorimetric assay kit (Cell Biolabs, San Diego, CA, US) was used to determine NO
25 bioavailability by measurement of the nitric oxide metabolites nitrite and nitrate (NO_x) in the
26 serum. Intra-assay and inter-assay coefficients of variation were 2.9% and 1.7% respectively.

1 **Serum 8-iso-Prostaglandin F2 α formation**

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1 Concentration of 8-iso-PGF2 α in serum was measured by validated enzyme immunoassay (EIA) method (DRG International, Springfield, NJ, USA). Intra-assay and inter-assay coefficients of variation were 5.8% and 5.0% respectively. Values were expressed as pmol/L.

5 **Myocardial function**

6 After 120 minutes and 5 days from the intervention, left ventricular end-diastolic volume (LVEDV), left ventricular end-systolic volume (LVESV) and ejection fraction (LVEF) were calculated by the biplane Simpson's rule, as recommended by the American Society of Echocardiography. The mean values of three measurements were used for statistical evaluation.

10 **Sample Size Calculation**

11 For the present preliminary analysis, the sample size calculation was estimated considering previous data available for 8-iso-PGF2 α levels [22]. A sample size of 25 patients undergoing GSH infusion provided an intervention study with 80% power to detect a 20% reduction in plasmatic 8-iso-PGF2- α levels measured at the end of successfully reperfusion with respect to the placebo group. We also assumed a 25% SD in each group.

16 **Statistical analysis**

17 Categorical variables were reported as counts (percentage) and continuous variables as means \pm standard deviation (SD). We tested the independence of categorical variables by χ^2 test and the normal distribution of continuous variables by Kolmogorov-Smirnov test. We used Student paired and unpaired t test, repeated measure ANOVA and Pearson product-moment correlation analysis to evaluate normally distributed continuous variables. Appropriate nonparametric tests (Mann-Whitney U test, Wilcoxon rank test and Spearman rank correlation test) were employed for all the other variables. As an overall nonparametric ANOVA, the Friedman test for the analysis of intragroup variations was used. In cases of significance, we compared pair related samples using the Wilcoxon test. The intergroup analysis was performed with the nonparametric Mann-Whitney U-test. Only two-tailed probabilities were used for testing statistical significance. Probability values <

0.05 were regarded as statistically significant. All calculations were made with the computer program STATISTICA 7 (StatSoft, Tulsa, OK, USA).

Results

Twenty-five patients randomly received GSH and 25 placebo. All patients completed the phases of the study (Figure 1). All patients had a TIMI flow grade equal to 0 or 1 requiring percutaneous treatment. Clinical and angiographic characteristics of patients are shown in Table 1 and 2. The baseline characteristics were well balanced between the two groups. In both groups, neither side effects during the infusion, nor adverse events during the short observation period were recorded.

Oxidative stress, antioxidant status and vascular function in peripheral samples. Biochemical data are summarized in Table 3. Baseline H_2O_2 and 8-iso-PGF 2α levels were similar between treated patients and controls. After PCI, a significant reduction of H_2O_2 production and 8-iso-PGF 2α levels was observed in GSH group as compared to controls (Figure 2A and 2B). Moreover, a significant increase in HBA and NO bioavailability was observed (Figure 2C and 2D).

At the 5 days from index procedure, a persistent significant reduction of H_2O_2 production and a sustained increase in HBA and NO bioavailability was observed in the GSH group as compared with controls (Figure 2A-D).

Serological markers of myocardial injury. Baseline cTnT mean values were similar between GSH and placebo groups (176.0 ± 20.9 pg/ml vs. 165.4 ± 20.9 pg/ml, $p=0.079$). At 6 hours, no changes in cTnT values were found in GSH-treated patients (172.1 ± 27.7 pg/ml vs. baseline, $p=0.065$). At 12 hours and 5 days after pPCI, GSH-treated patients showed a progressive decrease of cTnT levels (170.0 ± 44.7 pg/ml and 137.9 ± 23.7 pg/ml; $-21 \pm 23.1\%$, $p=0.009$ vs. baseline). Differently, a significant increase and persistence of high values of cTnT were observed in placebo group (T6, 169.9 ± 16.3 pg/ml, T12, 183.0 ± 34.8 pg/ml and T5d, 181.9 ± 18.0 pg/ml; $+12.4 \pm 23.1\%$, $p=0.029$ vs. baseline) (Figure 3A). A modest correlation between percentage changes of H_2O_2 and cTnT levels from baseline to 5 days was found in treated group (Figure 3B).

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3 1 **Myocardial Reperfusion indexes.** Post-procedural cTFC values did not show a statistically
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5 2 significant reduction between treated and control groups (20.7 ± 7.3 vs. 23.4 ± 5.1 , $p=0.156$).
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7 3 Interestingly, 6 patients (24%) in the placebo group and 15 (60%) patients in GSH group reached
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9 4 lower-risk (≤ 20 frames/s) cTFC class ($p=0.019$). After PCI, $\text{TMPG} \geq 2$ was assessed in 21 patients
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11 5 (84%) and 14 patients (56%) of the GSH and placebo groups, respectively ($p = 0.064$). Of note, 11
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13 6 patients (44%) of the GSH group only had $\text{TMPG}=3$ ($p=0.0002$ vs. controls). Post-reperfusion
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15 7 cTFC values showed a significant correlation with changes of 8-iso-PGF 2α ($R=0.55$, $p=0.012$)
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17 8 levels from baseline.

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21 9 **Myocardial function.** Myocardial function was not different between groups after either baseline
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23 10 or at discharge. There was no significant difference between groups regarding LVEF, LVEDV or
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25 11 LVESV at any time point (Table 4).

26 12 **Discussion**

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30 13 This pilot study demonstrates that in the setting of STEMI reperfusion the rapid onset and
31
32 14 prolonged antioxidant (scavenging) activity obtained by infusion of GSH before and after primary
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34 15 PCI reduces the oxidative stress markers. The improvement of the antioxidant status resulted in a
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36 16 significant decrease of cardiac troponin, marker of myocardial damage.

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40 17 Data from experimental and clinical studies suggest that following reperfusion myocardial
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42 18 cells death largely contributes to the final infarct size.[23,24] On the other hand, the extent of
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44 19 damaged myocardium is the most important predictor of adverse ventricular remodeling and it is
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46 20 linearly dependent upon the amount of myocardial salvage by and after reperfusion. Thus,
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48 21 attenuation of pro-oxidant state is an important goal in cardioprotective interventions.[25]
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50 22 Noteworthy, the serum of GSH treated patients showed a greater capacity to detoxify H_2O_2
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52 23 evaluated by the HBA, an assay that measure the percentage of H_2O_2 neutralized into the
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54 24 samples.[26] We found an early and considerable increase of HBA, with positive effects on
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56 25 myocardial cell survival, assessed by cTpT.
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3 1 Current evidences demonstrate that oxidant environment promotes cardiomyocyte death in
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5 2 the first few minutes of reflow suggesting the existence of a tight window of effective cardio-
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7 3 protection.[27,28] Therefore, ROS-induced injury may continue for weeks to months as a result of
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9 4 activation of programmed cell death. Our data have shown a persistent heightened oxidative status
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11 5 along with decreased scavenging activity in untreated patients. This behavior makes the duration of
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13 6 pharmacologic interventions a central point of cardio-protection strategies. In the present study,
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15 7 GSH infusion, starting just before reperfusion with subsequent administration up to 3 days after,
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17 8 promoted early and sustained increase of serum HBA with attenuated production of H₂O₂ which
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19 9 was highly related to progressive significant reduction of serological signs of myocardial injury. In
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21 10 addition, our data show a progressive significant decrease of serum cTnT release during the 5 days
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23 11 of reperfusion in the GSH-treated patients compared with the control group resulting in a 21%
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25 12 reduction of myocardial damage. Despite that, in our population, the systolic function was not
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27 13 different between groups after reperfusion, although a trend towards reduced LVEDV was observed
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29 14 in treated patients. A possible explanation relies on the fact that inside the area at risk variable
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31 15 amount of hibernated and stunned myocardium may coexist, thus affecting the prompt recovery of
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33 16 contractility after reperfusion.[29]

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40 17 Cells have a number of mechanisms for dealing with the toxic effects of oxygen. One of the
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42 18 most important is connected with the widely distributed tripeptide thiol glutathione.[16,30] In
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44 19 particular, the glutathione redox cycle is a more efficient antioxidant protective mechanism of the
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46 20 heart, which acts by maintaining thiol groups of enzymes and other proteins in their reduced state
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48 21 thus preventing cell membrane lipid peroxidation and limiting cardiomyocytes loss.[31]
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50 22 Furthermore, in our study, a close relation between reduced myocardial reperfusion and increased
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52 23 of 8-iso-PGF₂ α serum levels has been observed, suggesting that oxidative unbalance may be
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54 24 involved in microcirculation functional damage. As previously reported, impaired tissue-level
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56 25 perfusion develops within minutes of established acute revascularization of ischemic areas[32] and
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58 26 persists for at least 1 week.[33] In this context, there is robust evidence that ROS mediated

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3 1 isoprostanes production contributes importantly to the post-reperfusion microvascular
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5 2 impairment.[22,34] Current findings implement this observation by demonstrating a sustained
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7 3 production of isoprostanes up to 5 days after reperfusion thereby suggesting their contributory role
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9 4 in the pathogenesis and persistence of microvascular dysfunction that may affect myocardial cell
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11 5 survival. The infusion of GSH before and 24, 48, 72 hours after pPCI reduced isoprostanes serum
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13 6 levels and their reduction was linked to improvement of myocardial reperfusion indexes. Moreover,
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15 7 the increase in extracellular peroxide oxidants may reduce bioavailability of nitric oxide that is
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17 8 thought to contribute to promoting platelet hyperactivity and vasoconstriction.[13] In our study,
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19 9 GSH supplementation seems to have a role in preserving NO bioavailability and its vasodilator
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21 10 capacity at microcirculatory level.

26 11 **The Strengths and Limitations of the Study**

28 12 The positive effects on reperfusion indexes and on biochemical signs of myocardial necrosis
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30 13 suggest the value of prophylactic and prolonged GSH administration in preventing reperfusion
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32 14 injury. Thus, in patients undergoing pPCI the infusion of a powerful antioxidant scavenger, such as
33
34 15 GSH, may be useful to improve microcirculatory perfusion in order to further blunt the injury of
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36 16 myocardial cells.

40 17 **Some limitations deserve to be discussed.**

42 18 The small sample size of the study and the lack of morfologic assessment of both infarct size
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44 19 and microvascular obstruction extent between the two groups, actually, limit the clinical application
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46 20 of these findings. Within a defined area at risk, the manifestations of ischemia-reperfusion vascular
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48 21 injury go from reversible functional impairments to irreversible structural damage and contribute to
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50 22 final amount of infarct myocardium. In absence of morphologic imaging, the qualitative evaluation
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52 23 of GSH-induced improvement of myocardial reperfusion indexes, as assessed in our study, might
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54 24 only represent the effect of a preserved microcirculatory responsiveness to vasoactive substances
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56 25 (i.e. NO) but unable to limit the expansion of myocardial cell damage. Indeed, other mechanisms,
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58 26 such as interstitial edema and inflammatory reaction, which induce a sustained impairment of
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3 1 microvascular perfusion, may primarily act to increase the amount of irreversible injured
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5 2 myocardium thus promoting adverse ventricular remodeling.
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8 3 In conclusion, in this pilot study, we have shown that a short-term prophylactic GSH
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10 4 infusion mitigates the negative effects of the excessive and persistent H₂O₂ formation on myocardial
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12 5 cells. The findings of the present study require to be confirmed through an adequately powered
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14 6 STEMI population. A larger trial with a prolonged follow-up for evaluation of clinical endpoints is
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17 7 needed to confirm the role of GSH administration as cardioprotective therapy.
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9 5 **Figure 1. CONSORT flowchart**
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13 7 **Figure 2.** H₂O₂ production (A), 8-iso-PGF₂α formation (B), hydrogen peroxide breakdown activity
14 (HBA) (C) and NO bioavailability (D) at baseline, after 2 hours (T2h) and at the 5 days (T5d) from
15 the PCI in patients received GSH (n=25, dashed line) or placebo (n=25, continuous line).
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20 10 Data are expressed as mean±SEM (***p <0.001, **p <0.01, *p <0.05).
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25 12 **Figure 3.** cTpT levels (A) at baseline, after 6 hours (T6h), 12 hours (T12h) and at the 5 days (T5d)
26 from the PCI in patients received GSH (n=25, dashed line) or placebo (n=25, continuous line). Data
27 13
28 are expressed as mean±SEM (***p<0.0001 vs. T0, *p<0.05 vs. T0, \$p<0.05 between groups).
29 14
30 15 Linear correlation between % Δ cTpT and % Δ H₂O₂ in GSH treated group (B).
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Contributors

GTa, EM led on the conception, design and writing of the study with substantial contributions to the design, writing, critical review of intellectual content; GTr, AA, AP, NV, RM, enrollment of patients; RC, VC, CN, provided laboratory analyses; VR, SB, provided further essential statistical advice and expertise on the study protocol; FB, LL, MP, CG, providing expert clinical support; AG, MD, made substantial contributions to the trial design and management.

Ethics and dissemination

Italian Medicines Agency (AIFA) authorization and single Ethic Committee approval has been obtained for all the centers participating the study. Dissemination of results will be via peer-reviewed research publications both online and in print, conference presentations, posters, patient forums and Trust bulletins.

Data Availability Statement

The data set is available on request from the corresponding author.

Conflicts of Interest

Authors declare that there are not conflicts of interest.

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151 **Table 1. Clinical characteristics of the study population**
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Variables	GSH group (n=25)	Placebo group (n=25)	P value
Age (y, mean±SD)	66 ± 10.7	66.9 ± 9.1	0.74
Male, n (%)	15 (60)	13 (52)	0.98
Body-mass index§ (mean+SD)	26.9 ± 3.9	20 ± 3.8	0.38
Killip class ≥ 3, n (%)	2 (8)	0 (0)	0.47
Diabetes Mellitus, n (%)	5 (20)	5 (20)	1
Hypertension, n (%)	14 (56)	17 (68)	0.56
Dyslipidemia, n (%)	11 (44)	13 (52)	0.77
Statin use, n (%)	8 (32)	8 (32)	1
Smokers, n (%)	17 (68)	13 (52)	0.38

§ The body-mass index is the weight in kilograms divided by the square of the height in meters.

Table 2. Angiographic parameters

Variables	GSH group (n=25)	Placebo group (n=25)	P value
Ischemia time# (min; mean \pm SD)	286 \pm 88	270 \pm 96	0.85
Thrombus Burden \geq 3, n (%)	12 (48)	11 (44)	0.77
Thrombus aspiration, n (%)	13 (52)	12 (48)	0.87
GP IIb/IIIa inhibitors, n (%)	2 (8)	3 (12)	0.63
MVD, n (%)	13 (52)	11 (44)	0.77
2 vessels,	8 (32)	5 (20)	0.89
3 vessels,	5 (20)	6 (24)	
Staged PCI, n (%)	9 (36)	5 (20)	
IRA:			
LAD, n (%)	10 (40)	9 (36)	0.77
LCx, n (%)	5 (20)	6 (24)	0.73
RCA, n (%)	10 (40)	10 (40)	1

PCI = percutaneous coronary intervention; IRA = infarct related coronary artery; LAD = left anterior descending coronary artery; LCx = left circumflex coronary artery; RCA = Right coronary artery. #ischemia time was defined as the timing between symptom onset and balloon inflation.

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3 **Table 3. Biochemical data**
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	Baseline			Reperfusion 2h			Follow-up (5 days)		
Variable	GSH	Placebo	p	GSH	Placebo	p	GSH	Placebo	p
H₂O₂ μM, mean±SD	40.6±8.4	43.6±11.6	0.305	28.4±12	42.8±14.1	0.0003	24±7	39.5±17.3	0.0001
Δ				-12.1±15.2	-0.7±17.9	0.03	-16.6±11.0	-4.1±20.14	0.009
8-iso-PGF2α pmol/L, mean±SD	214.6±81.1	211.9±92.1	0.91	163.6±44.7	217.6±51.6	0.0003	159.9±34.2	213.1±50.9	0.0001
Δ				-50.9±92.9	-3.3±1.29	0.02	-54.6±62.1	-1.2±115.7	0.02
HBA %, mean±SD	43.6±7.4	43.4±11.9	0.94	57.9±8.6	43.9±8.7	0.0001	62.9±10.5	45.2±13.0	0.0001
Δ				+14.9±5.5	+0.4±14.9	0.0004	+19.4±10.2	+1.8±17.1	0.0001
NO μM, mean±SD	16.3±5.7	16.5±4.7	0.89	27.7±7.2	22.4±10	0.0356	35.5±8.1	23.5±15.5	0.0013
Δ				+11.4±6.8	+5.8±10.5	0.05	+19.2±9.7	+7.0±14.7	0.002

45 **GSH** = reduced Glutathione; **H₂O₂** = Hydrogen Peroxide; **8-iso-PGF2α** = 8-iso-Prostaglandin-F2α;

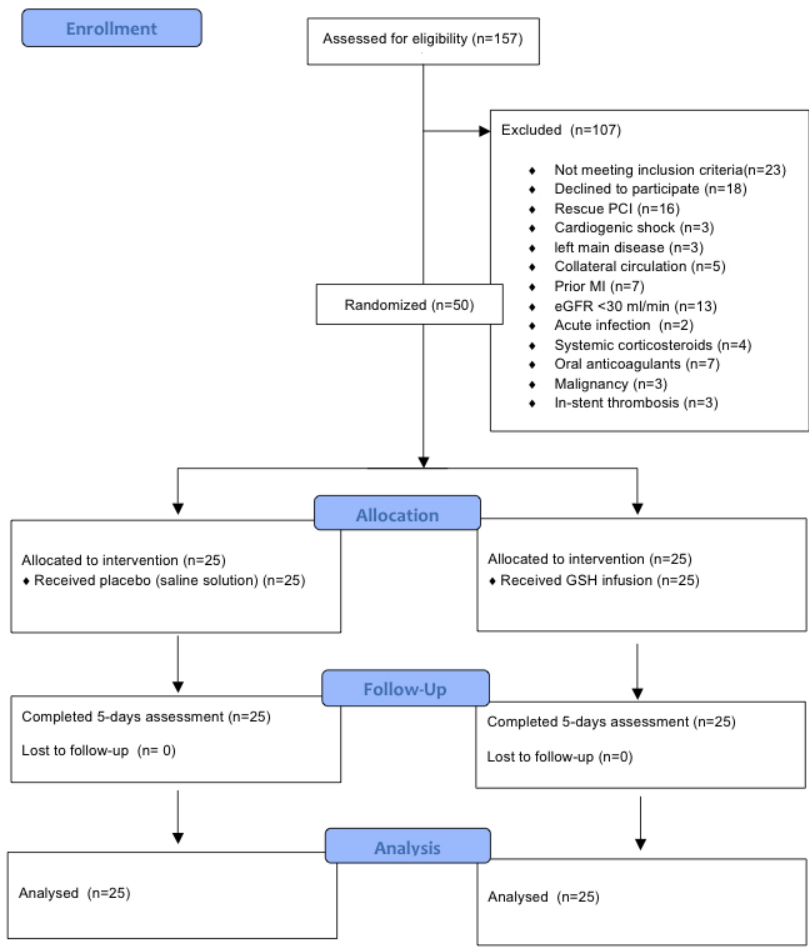
46 **HBA** = H₂O₂ break-down activity

Table 4. Left Ventricular echocardiographic parameters at baseline and at follow-up

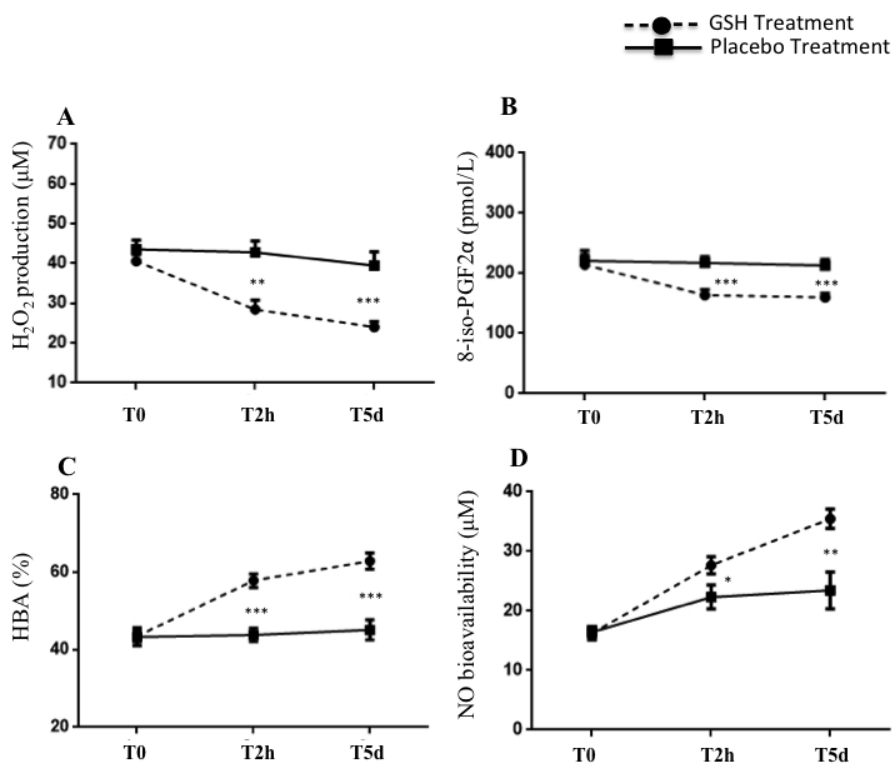
Echo parameters	Placebo (n= 25)	GSH (n=25)	P-value
Baseline			
LVEDV (mL/m²)	121.3 ± 17.2	124.4 ± 22.3	0.44
LVESV (mL/m²)	65.4 ± 11.3	66.3± 13.2	0.91
LVEF (%)	47.5 ± 4.9	46.9 ± 4.8	0.42
Follow-up			
LVEDV (mL/m²)	118.1 ± 17.8	113.2 ± 14.1	0.42
LVESV (mL/m²)	60.9 ± 10.7	58.8± 12.5	0.91
LVEF (%)	49.1 ± 3.2	49.8 ± 3.7	0.42
LVEDV = left ventricular end-diastolic volume; LVESV = left ventricular end-systolic volume; LVEF = left ventricular ejection fraction-diastolic volume			

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CONSORT flowchart

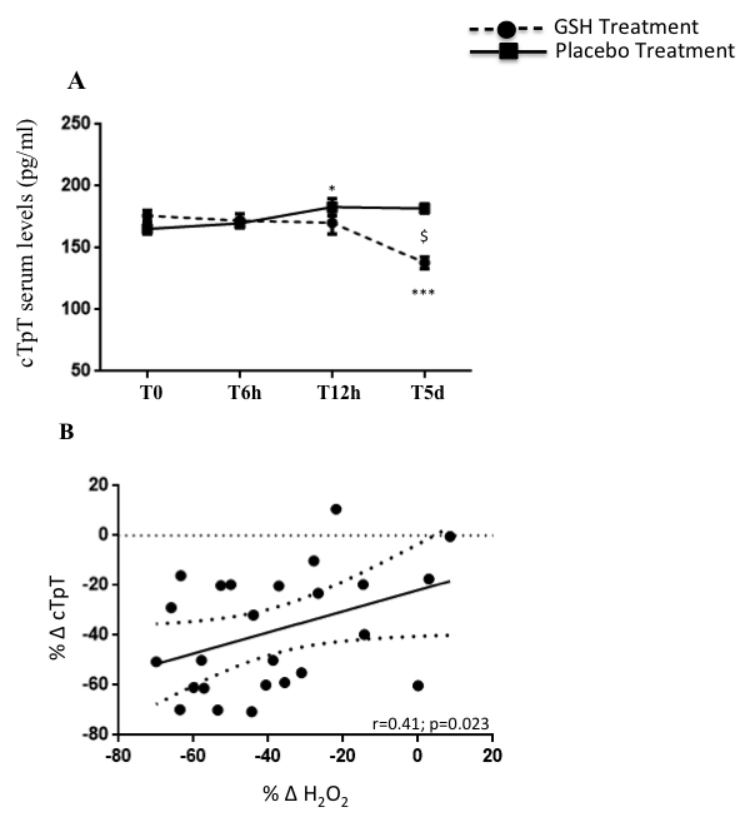


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3 **EudraCT Number: 2014-004486-25**

4 **Sponsor's Protocol Code Number: GSH2014**

5 National Competent Authority: Italy - Italian Medicines Agency

6 Clinical Trial Type: EEA CTA

7 Trial Status: submitted data of the pilot study. The trial is ongoing.

8 Date on which this record was first entered in the EudraCT database: 2014-12-04

9 Link: <https://www.clinicaltrialsregister.eu/ctr-search/trial/2014-004486-25/IT/>

12
13 **A. Protocol Information**

14 Member State Concerned: Italy - Italian Medicines Agency

15 EudraCT number: 2014-004486-25

16 Full title of the trial: Prevention of the reperfusion myocardial damage in patients with acute
17 myocardial infarct (STEMI) submitted to primary PCI through infusion of intravenous glutathione.

18 Sponsor's protocol code number: GSH2014

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21 **B. Sponsor Information**

22 Sponsor 1: University Hospital "Policlinico Umberto I"

23 Name of organization providing support: University Hospital "Policlinico Umberto I", Rome, Italy.

24 Functional name of contact point: Enrico Mangieri, University Hospital "Policlinico Umberto I".

25 Viale del Policlinico, 155 – Rome, Post code: 00161, Italy

26 E-mail: enrico.mangieri@uniroma1.it

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29 **D. IMP Identification**

30 *IMP to be used in the trial has a marketing authorisation: Yes*

31 *Trade name: TAD*

32 Name of the Marketing Authorisation holder: Biomedica Foscama Group S.p.A.

33 Country which granted the Marketing Authorisation: Italy

34 Pharmaceutical form: Powder and solvent for solution for infusion

35 Routes of administration for this IMP: Intravenous use

36 *Information on Placebo*

37 Pharmaceutical form of the placebo: saline solution

38 Route of administration of the placebo: Intravenous use

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41 **E. General Information on the Trial**

42 Medical condition or disease under investigation

43 Medical condition(s) being investigated: ST-Segment Elevation Myocardial Infarction (STEMI).

44 Medical condition in easily understood language: acute myocardial infarct

45 Therapeutic area: Diseases [C] - Cardiovascular Diseases [C14]

46 Objective of the trial

47 Main objective of the trial: To verify if the intravenous infusion of "Glutathione Sodium Salt" it is
48 able to reduce the level of oxidative state in the area of myocardial infarction.

49 Secondary objectives of the trial: To verify if the intravenous infusion of "Glutathione Sodium Salt"
50 during the procedures of primary PCI it is able to limit the extension of the ischemic area, to
51 reduce the incidence of the no-reflow, to improve the degree of myocardial blush and to decrease
52 the indexes of suffering post-procedural ischemia (ST elevation; release of myocardial necrosis
53 markers).

54 Principal inclusion criteria: STEMI patients submitted to p-PCI up to 12 hours.

55 Age ≥ 18 years. Women and Men. Signed informed consent

Principal exclusion criteria:

Patients with cardiac arrest, ventricular fibrillation, cardiogenic shock, stent thrombosis, previous acute myocardial infarction, or angina within 48 hours before infarction were not included in the study. Patients with evidence of coronary collaterals (2-3 Rentrop) to the region at risk on initial coronary angiography (at the time of admission) will be excluded. Moreover, patients with EF $\leq 30\%$, impaired renal function (creatinine > 3.0 mg/dl), recipient of heart transplant, a life expectancy less than 12 months, has known allergies to aspirin, clopidogrel bisulfate, heparin, contrast media or stainless steel that cannot be managed medically were excluded. Patient needs therapy with warfarin or currently participating in an investigational drug or another device study were not considered enrolling.

End points

Primary end point(s): The primary endpoint will consist in the assessment of the effects of the infusion of "Glutathione Sodium Salt" on the reduction of the oxidative markers and inflammation after PCI.

Timepoint(s) of clinical evaluation of this end point: before, 2 hour and 5 days from the p-PCI

Secondary end point(s): The secondary endpoint will include: (1) the assessment of the variations of the corrected TIMI frame count (cTFC) and the TIMI Myocardial Perfusion Grade (TMPG) after p-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 occurrence of a new revascularization, up to 6 months after the procedure.

Medical Doctors don't have the knowledge both about the possible infusion of the Glutathione Sodium Salt, in the examined patient, then others clinical data.

Moreover, serological levels of Troponin and creatinine will be measured before the p-PCI and after the procedure (2, 6, 12 and 24 hours).

Besides, through 2D Echocardiography with Simpson's biplane method the FE will be calculate at admission and after hospital discharge.

If clinical-instrumental signs of ischemia will rise up, the patient will be submitted to a new angiography.

Definition of the end of the trial and justification where it is not the last visit of the last subject undergoing the trial: LVLS or telephonic contact

Population of Trial Subjects

Trial has subjects under 18: No

Adults (18-64 years): Yes

Number of subjects for this age range: 30

Elderly (≥ 65 years): Yes

Number of subjects for this age range: 60

Female: Yes

Male: Yes

Patients: Yes

Specific vulnerable populations: Yes

Women of childbearing potential not using contraception: Yes

Women of child-bearing potential using contraception: Yes

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3 Pregnant women: No
4 Nursing women: No
5 Emergency situation: No
6 Subjects incapable of giving consent personally: No
7 Planned number of subjects to be included: 90
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10 **F. Investigator Networks to be involved in the Trial**

11 N. Review by the Competent Authority or Ethics Committee in the country concerned
12 N. Competent Authority Decision: Authorised
13 N. Date of Competent Authority Decision: 2015-01-13
14 N. Ethics Committee Opinion of the trial application: Favourable
15 N. Date of Ethics Committee Opinion: 2015-02-12
16 N. Centers involved in the study: Department of Heart and Great Vessel "A. Reale", Sapienza
17 University of Rome (coordinator centre) - "Santa Maria" Terni Hospital - "San Giovanni
18 Evangelista" Tivoli Hospital, all in Italy.
19 P. End of Trial Status: analyzed as pilot study the first 50 enrolled patients. Ongoing.
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CONSORT 2010 checklist of information to include when reporting a randomised trial*

Section/Topic	Item No	Checklist item	Reported on page No
Title and abstract			
	1a	Identification as a randomised trial in the title	1
	1b	Structured summary of trial design, methods, results, and conclusions (for specific guidance see CONSORT for abstracts)	2
Introduction			
Background and objectives	2a	Scientific background and explanation of rationale	4
	2b	Specific objectives or hypotheses	5
Methods			
Trial design	3a	Description of trial design (such as parallel, factorial) including allocation ratio	6
	3b	Important changes to methods after trial commencement (such as eligibility criteria), with reasons	NA
Participants	4a	Eligibility criteria for participants	6
	4b	Settings and locations where the data were collected	7
Interventions	5	The interventions for each group with sufficient details to allow replication, including how and when they were actually administered	7
Outcomes	6a	Completely defined pre-specified primary and secondary outcome measures, including how and when they were assessed	7-8
	6b	Any changes to trial outcomes after the trial commenced, with reasons	NA
Sample size	7a	How sample size was determined	9
	7b	When applicable, explanation of any interim analyses and stopping guidelines	9
Randomisation:			
Sequence generation	8a	Method used to generate the random allocation sequence	7
	8b	Type of randomisation; details of any restriction (such as blocking and block size)	7
Allocation concealment mechanism	9	Mechanism used to implement the random allocation sequence (such as sequentially numbered containers), describing any steps taken to conceal the sequence until interventions were assigned	7
Implementation	10	Who generated the random allocation sequence, who enrolled participants, and who assigned participants to interventions	7
Blinding	11a	If done, who was blinded after assignment to interventions (for example, participants, care providers, those	7

		assessing outcomes) and how	
	11b	If relevant, description of the similarity of interventions	NA
Statistical methods	12a	Statistical methods used to compare groups for primary and secondary outcomes	9-10
	12b	Methods for additional analyses, such as subgroup analyses and adjusted analyses	NA
Results			
Participant flow (a diagram is strongly recommended)	13a	For each group, the numbers of participants who were randomly assigned, received intended treatment, and were analysed for the primary outcome	7
	13b	For each group, losses and exclusions after randomisation, together with reasons	7
Recruitment	14a	Dates defining the periods of recruitment and follow-up	6
	14b	Why the trial ended or was stopped	NA
Baseline data	15	A table showing baseline demographic and clinical characteristics for each group	21-22
Numbers analysed	16	For each group, number of participants (denominator) included in each analysis and whether the analysis was by original assigned groups	10
Outcomes and estimation	17a	For each primary and secondary outcome, results for each group, and the estimated effect size and its precision (such as 95% confidence interval)	10-11
	17b	For binary outcomes, presentation of both absolute and relative effect sizes is recommended	NA
Ancillary analyses	18	Results of any other analyses performed, including subgroup analyses and adjusted analyses, distinguishing pre-specified from exploratory	NA
Harms	19	All important harms or unintended effects in each group (for specific guidance see CONSORT for harms)	10
Discussion			
Limitations	20	Trial limitations, addressing sources of potential bias, imprecision, and, if relevant, multiplicity of analyses	13-14
Generalisability	21	Generalisability (external validity, applicability) of the trial findings	14
Interpretation	22	Interpretation consistent with results, balancing benefits and harms, and considering other relevant evidence	14
Other information			
Registration	23	Registration number and name of trial registry	5
Protocol	24	Where the full trial protocol can be accessed, if available	Supplementary file
Funding	25	Sources of funding and other support (such as supply of drugs), role of funders	16

*We strongly recommend reading this statement in conjunction with the CONSORT 2010 Explanation and Elaboration for important clarifications on all the items. If relevant, we also recommend reading CONSORT extensions for cluster randomised trials, non-inferiority and equivalence trials, non-pharmacological treatments, herbal interventions, and pragmatic trials. Additional extensions are forthcoming: for those and for up to date references relevant to this checklist, see www.consort-statement.org.