

ONLINE DATA SUPPLEMENT

PROTOCOL: “Nitric Oxide to Reduce Acute Kidney Injury in Patients with Pre-existing Endothelial Dysfunction Requiring Prolonged Cardiopulmonary Bypass: A Randomized Clinical Trial”

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Dates of study protocol to Massachusetts General Hospital Investigational Review Board:

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Synopsis and Study Schema

Title	Peri-operative Nitric Oxide Therapy to Reduce Acute Kidney Injury in Cardiac Surgery Patients with Endothelial Dysfunction Requiring Prolonged Cardiopulmonary Bypass: A Randomized Clinical Trial.
Study Objective(s)	To determine whether Nitric Oxide (NO) administered during cardio-pulmonary bypass (CPB) and after prolonged CPB reduces clinically significant acute renal injury (AKI) (in the first 7 days after surgery) and other major short-term complications (at 6 weeks and 90 days) and whether the benefits are maintained for one year in patients undergoing prolonged CPB for cardiac surgery.
Study Design	A single academic center, prospective, randomized, double-blind controlled trial comparing treatment with NO versus nitrogen (N ₂) in adult patients undergoing prolonged CPB heart surgery.

Study Population

Main selection criteria

Inclusion	<ol style="list-style-type: none">1. Provide written informed consent2. Age ≥ 18 years of age3. Elective cardiac or aortic surgery with estimated CPB >90 minutes4. Clinical evidence of endothelial dysfunction assessed by a specifically designed questionnaire (Figure 3)5. Stable pre-operative renal function without evidence of a plasma creatinine increase of ≥ 0.3 mg/dL within 3 months of study entry and without receiving renal replacement therapy (RRT)
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Exclusion

1. eGFR less than 30 ml/min/1.73 m²
2. Emergent cardiac surgery
3. Life expectancy < 1 year at the time of enrollment
4. Mean pulmonary artery pressure ≥ 40 mm Hg and pulmonary vascular resistance > 4 Wood Units
5. Left ventricular ejection fraction < 30% by echocardiography obtained within three months of enrollment
6. Hemodynamic instability as defined by a systolic blood pressure < 90mmHg
7. Administration of one or more Packed Red Blood Cell (PRBC) transfusions in the week prior to enrollment
8. X-ray contrast infusion less than 48h before surgery
9. Evidence of hemolysis from any other origin:
 - a. Intravascular:
 - i. Intrinsic RBC defects leading to hemolytic anemia (eg, enzyme deficiencies, hemoglobinopathies, membrane defects)

ii. Extrinsic: liver disease, hypersplenism, infections (eg, bartonella, babesia, malaria), treatment with oxidizing exogenous agents (eg, dapsone, nitrites, aniline dyes), exposure to other hemolytic agents (eg, lead, snake and spider bites), lymphocyte leukemia, autoimmune hemolytic disorders

b. Extravascular:

Infection (eg, clostridial sepsis, severe malaria), paroxysmal cold hemoglobinuria, cold agglutinin disease, paroxysmal nocturnal hemoglobinuria, IV infusion of Rho(D) immune globulin, IV infusion of hypotonic solutions

Total expected number of subjects: 250

Recruiting Center	Departments of Anesthesia and Cardiac Surgery at the Massachusetts General Hospital, Boston, MA
Study Drug or Intervention	Patients will be randomized into one of two groups; one group will receive NO during CPB and for 24-hours after the procedure, the other group will receive N ₂ .
Evaluation Criteria	The occurrence of AKI and any other organ damage when evaluated by the SOFA score ¹ , prolonged ventilation or mortality reduction.
Primary Endpoints	Difference in the incidence of AKI between the control group (receiving N ₂) versus the study group (receiving NO). AKI is defined by abrupt (within 48h) reduction in kidney function correlated to an absolute increase in serum creatinine of 0.3 mg/dL or more ($\geq 26.4 \mu\text{mol/L}$) or a percentage increase in serum creatinine of 50% or more (1.5-fold from baseline) at any time during the first 7 days after surgery or, finally, a reduction in urine output with a documented oliguria of $< 0.5 \text{ ml/Kg/h}$ for $> 6\text{h}$. ²
Secondary Endpoints	<p><u>Secondary renal endpoints:</u> Difference between the groups of AKI severity as reflected by the changes of serum creatinine and urinary output (KDIGO stages)². Difference between groups of incidence and severity (KDOQI stages 3, 4, 5)³ of Chronic Kidney Disease (CKD) at 1 year after surgery. New requirement for RRT following AKI during hospitalization and at 6 weeks, 90 days and one year after surgery. Incidence of Major Adverse Kidney Events (MAKE)⁴ at 6 weeks, and 1 year after surgery.</p> <p><u>Secondary non-renal endpoints:</u> Incidence and severity of other single organ dysfunction (SOFA), prolonged cardiovascular support, Vasoactive-Inotropic Score (VIS)^{72,73}, duration of mechanical ventilation. Evaluation of ICU and hospital length of stay.</p>
Exploratory Endpoints	Incidence and severity of AKI related to the presence of CKD at baseline, duration of CPB, duration of aortic cross-clamp, levels of free Hb, levels

of NO consumption, pulmonary pressure at baseline, cardiovascular risks associated with endothelial dysfunction, scheduled procedure and EuroSCORE II^{5,6}. Differences between groups of delirium^{7,8}.

Safety Endpoints

Difference between groups of mortality at 6 weeks, 90 days and 1 year after surgery and stratification according to the initial EuroSCORE II. Differences between the two groups of transfusions with plasma and stored or autologous red blood cells (RBCs) and bleeding. Incidence of non-fatal stroke. Incidence of peri-operative and non-perioperative non-fatal myocardial infarction¹⁰. Continuous Monitoring of MetHb with a non-invasive co-oximetry^{11,12} monitor in order to prevent methemoglobinemia (defined as MetHb > 5%). Continuous monitoring of Nitrogen Dioxide (NO₂) in inhaled breath (threshold level < 5 ppm) to prevent reaching dangerously high levels (goal < 5 ppm). Differences between groups of post-operative infections, cardiac arrhythmias and other non-cardiac post-operative complications.

Statistical Considerations

Intention to treat analysis and per protocol analysis will be carried out. Comparison of the proportion of patients developing AKI. The sample size of this trial was calculated based on the primary endpoint: the reduction of AKI incidence. In recent reports from US and European studies at major academic centers, AKI incidence has been reported to be between 50 to 60% after prolonged CPB. In our recent trial of NO in cardiac surgery in Xian, China, the sample size was calculated to find a 30% reduction in the incidence of AKI (n=212 in that trial). We estimate a greater (35%) reduction in the incidence of AKI because we anticipate that our predominantly Caucasian population at MGH with endothelial dysfunction could be more assisted by the beneficial therapeutic properties of NO. Thus, in the NO group, the incidence of AKI is expected to decrease from 55% to 35.75%. The sample size needed to detect a difference, assuming an alpha error of 0.05 (two-sided test) and a study power of 0.8, is 114 patients per group. In order to account for possible dropouts, we increased our sample size by 10%. We will enroll 250 patients.

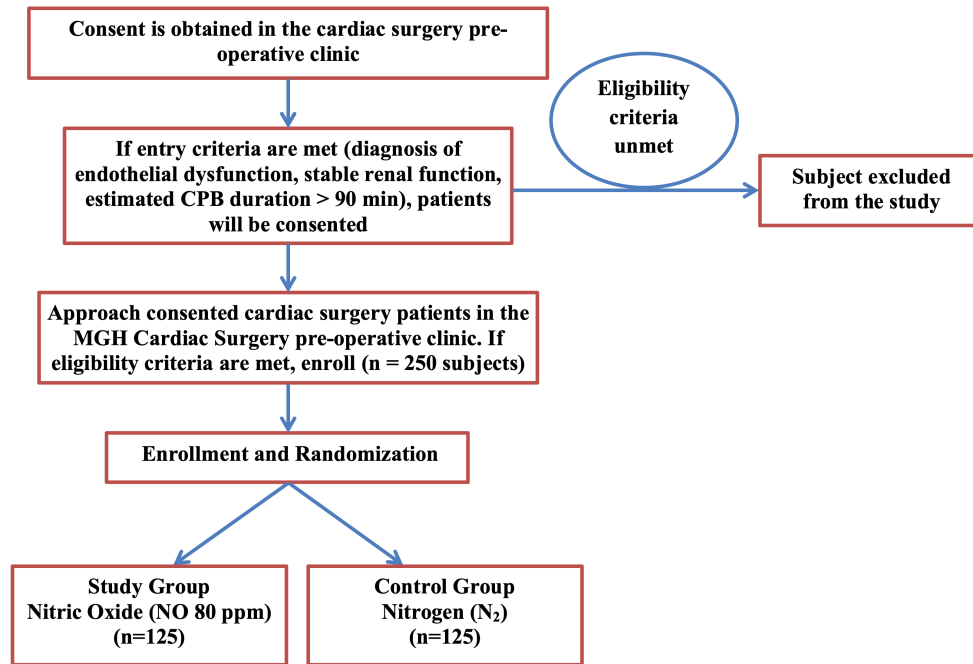
Duration of Study Period

Study participants will be followed up for 1 year after cardiac surgery.

Data Safety Monitoring Board

- Michael Fitzsimons, MD (Site DSMB Member). Division Chief of Cardiac Anesthesia at Massachusetts General Hospital, Boston, MA
- Francesco Nordio, PhD (DSMB Independent Statistician). Lead Biostatistician, TIMI Study Group, Brigham Women's Hospital, Boston, MA
- John Prowle, MA MB BChir MSc MD FRCP FFICM (DSMB Chair). Clinical reader in Critical Care Nephrology in the Critical Care and Peri-operative Medicine Research Group at William Harvey Research Institute, Queen Mary University of London, London, UK.

Figure 1. Study schema



I. BACKGROUND AND SIGNIFICANCE.

a. Historical background and rationale behind the proposed research

Background and significance. Acute kidney injury (AKI) is a common serious complication of cardiac surgical procedures that require prolonged (>90 minutes) cardiopulmonary bypass (CPB). The presence of AKI after CPB is associated with increased morbidity and mortality. The incidence of AKI after CPB depends on the type of operation, the duration of CPB and patient characteristics that adversely affect vascular function¹³⁻¹⁵. In a recent study from Duke University, 54% of the 4,217 adult patients who underwent coronary artery bypass grafting surgery developed AKI. Patients undergoing valve surgery, or a combination of both procedures, have a higher rate of AKI (as high as 60-70%)¹⁶⁻²⁷. Renal replacement therapy (RRT) is employed to treat the most severe cases of AKI. In a recent European trial involving patients without pre-existing kidney disease who underwent cardiac surgery with CPB, RRT was required in 16% of the participants¹⁸. One important risk factor for the development of surgery-associated AKI is pre-existing renal disease. Compared to patients without renal impairment, patients with a pre-existing reduced creatinine clearance have both a significantly greater incidence of AKI and mortality after cardiac surgery^{13, 22}. Hemolysis is an important risk factor for post-surgery AKI. The increased level of plasma hemoglobin (Hb) at the end of CPB closely correlates with the incidence and severity of AKI²⁸⁻³³. During hemolysis, Hb is released into the circulation in the form of oxyhemoglobin (Oxy-Hb), which depletes vascular nitric oxide (NO) via the dioxygenation reaction to form methemoglobin (Met-Hb)³⁴⁻³⁶. Endogenous NO is a potent dilator of vascular smooth muscle and NO depletion by plasma-free hemoglobin produces vasoconstriction, impaired tissue perfusion and inflammation³⁷⁻⁴². Under normal physiological conditions, a decrease in vascular NO bioavailability leads to increased production of NO by endothelial NO synthase (eNOS), the enzyme responsible for producing vascular NO. This sensitive feedback mechanism is impaired by endothelial dysfunction, which occurs in patients with atherosclerosis, peripheral vascular disease, hypertension, obesity, and diabetes⁴³⁻⁴⁵. The inability to increase eNOS activity by patients with endothelial dysfunction results in reduced blood vessel dilation, and, consequently, reduced tissue perfusion^{37, 38, 40, 41, 46-48}. Therapeutic exogenous administration of NO gas oxidizes free plasma Oxy-Hb to Met-Hb; the latter Fe⁺³ species are unable to deplete plasma NO^{33-36, 43, 49}.

Hypothesis of the study. We hypothesize that administration of NO during and for 24 h after prolonged CPB will convert free plasma Hb to metHb and prevent NO scavenging by free plasma Oxy-Hb and preserve kidney function, even in patients with evidence of endothelial dysfunction.

Rationale behind the proposed research. 1) Increased plasma-free Hb, a result of hemolysis, is associated with AKI following cardiac surgery with CPB. Many factors contribute to increased plasma Hb in cardiac surgery patients, including prolonged CPB, transfusion of autologous red blood cells

(RBCs) recovered using intraoperative cell salvage devices, and the transfusion of stored RBCs^{44, 50, 51}. The levels of plasma Hb are correlated with the incidence and severity of AKI²⁸⁻³³. Hemolysis has also been identified as an important contributor to AKI after cardiac surgery^{16, 19, 52}.

2) Plasma Hb, in the form of Oxy-Hb, reacts with vascular NO to form Met-Hb and normal endothelium compensates for this reaction by increasing NO production. In the vascular system, NO is produced by endothelial cells and regulates blood flow to tissue by decreasing vascular muscle tone. During hemolysis, however, NO reacts with circulating plasma Oxy-Hb in a rapid and irreversible dioxygenation reaction that produces nitrate and Met-Hb, thereby removing NO^{34-36, 42, 43, 49}. Under the IRB study protocol #2010P000961, we have shown that healthy subjects will increase plasma levels of nitrite, a precursor of NO, in response to an acute and limited increase in plasma consumption of NO⁵³. The result of increased NO production is to maintain intact regulation of blood perfusion. In cases of substantial hemolysis, as may occur during prolonged CPB, even healthy endothelium may be unable to produce sufficient NO. Exogenous administration of NO gas may then be necessary to convert plasma Oxy-Hb to Met-Hb and prevent the scavenging of endogenous NO.

3) Compared to normal endothelium, dysfunctional endothelium is less capable of producing NO. In the United States, cardiac surgery patients often have pre-existing endothelial dysfunction associated with peripheral vascular disease, hypertension, diabetes and obesity^{16-23, 25, 27}. No treatment has been tested to supplement insufficient endothelial NO production during and after hemolysis. Endothelial function may be evaluated in human brachial and digital arteries by assessing vasodilation in response to stimuli (such as brief ischemic periods) known to enhance the activity of eNOS, thereby increasing the production of endothelial NO. Peripheral arterial tonometry is a validated method to assess endothelial function^{38, 43, 54}.

4) Depletion of vascular NO causes vasoconstriction. We and other investigators have observed in pre-clinical studies and human studies that during acute hemolysis, plasma NO consumption, measured by an increase in plasma NO consumption, is increased and causes vasoconstriction, characterized by an acute increase in both systemic and pulmonary vascular resistance (IRB protocol #2010P000961, IRB #2011P000700, IRB #2013P000911, IRB #2013P002596). Intravenous administration of Hb solutions to animals, healthy volunteers and patients is associated with a dose-dependent increase in systemic and pulmonary arterial pressure^{38-40, 44, 47, 48, 55-59}.

5) Extra-pulmonary effects of exogenous NO administration: exogenous NO prevents plasma Oxy-Hb from consuming vascular NO. Inhaled NO is FDA-approved for treating pulmonary hypertension of the newborn and, at the Massachusetts General Hospital, is also used to treat adult pulmonary hypertension and right heart failure. When NO is breathed, it diffuses rapidly across the alveolar-capillary membrane, activating soluble guanylate cyclase, which converts guanosine-5'-triphosphate to cyclic guanosine monophosphate and relaxes the smooth muscle of pulmonary vessels⁶⁰. Over the past 2 decades, the

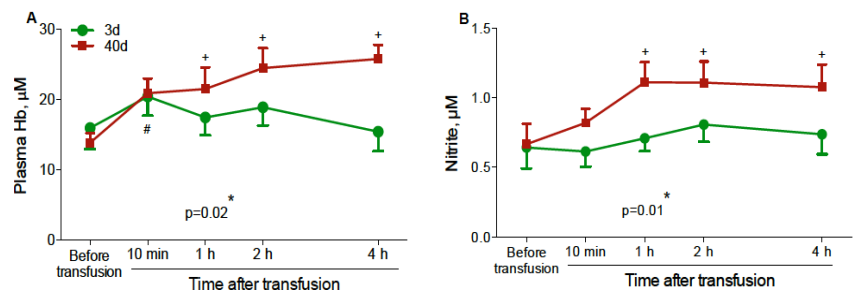
extra-pulmonary effects of NO have been recognized. Once NO enters the pulmonary circulation, NO may also bind to Hb to form S-nitrosoHb, or modify other circulating proteins. In IRB protocol #2010P000961, we have shown in healthy volunteers receiving autologous blood transfusions that NO increases plasma NO metabolites such as nitrate and, in a small amount, nitrite. In addition, breathing NO can oxidize plasma Oxy-Hb to Met-Hb, thereby reducing plasma NO consumption (IRB #2011P000700)^{34, 38, 42, 61-63}.

6) Measurements of markers of tubular damage may assist standard assessments in identifying and characterizing early renal dysfunction. The diagnosis of AKI is based on the KDIGO (Acute Kidney Injury Network) criteria^{2, 64, 65}. Both tools rely on the detection of an elevated level of plasma creatinine, which by itself has a limited value in terms of diagnosing early kidney injury after cardiac surgery. Elevated plasma creatinine occurs several hours after the kidney is injured, precluding timely intervention. A second potential limitation of using the serum creatinine level to detect renal injury is that creatinine is a functional marker of glomerular filtration and not of renal tubular injury, which is one of the key features of post-cardiac surgery AKI. The recent introduction of several plasma and urinary markers of tubular damage may allow earlier detection of AKI^{18, 66-68}. The ability to measure early markers of kidney damage facilitates clinical studies evaluating the effectiveness of a treatment that may prevent AKI during cardiac surgery with CPB. In this proposed trial, we will define AKI based on the KDIGO criteria². In addition, we will measure plasma and urinary renal biomarkers to help detect the onset of AKI and the severity of renal tubular injury.

b. Previous pre-clinical or clinical studies leading up to and supporting the proposed research
Preliminary studies at the Massachusetts General Hospital.

The rationale and the methods for the proposed research build on our prior Mass General Brigham IRB-approved studies: #2010P000961, IRB #2011P000700, IRB #2013P000911, IRB #2013P002596. Several of these studies were published in peer-reviewed clinical journals^{37, 38, 47, 48, 53, 56, 57, 60}.

1) IRB #2010P000961: Hemolysis increases NO synthesis in healthy endothelium. We enrolled 10 healthy adults at the MGH Blood Bank to receive one unit of 3-day stored autologous leukoreduced blood (not hemolyzed) followed in two weeks by an infusion of one unit of 40-day stored autologous leukoreduced blood (with up to 1% hemolysis). After transfusion of the 40-day, but not the 3-day stored blood, plasma Hb and nitrite levels increased significantly (Fig. 1A, 1B). The



results suggest that hemolysis, as demonstrated by increased plasma Hb, triggers a natural compensatory mechanism, possibly of healthy endothelium, increasing endogenous NO production by eNOS⁵³.

2) IRB #2011P000700: Hemolysis increases plasma consumption of NO and causes pulmonary vasoconstriction in volunteers with endothelial dysfunction. Breathing NO inhibits further consumption of plasma NO and prevents vasoconstriction. In a second study at the MGH blood bank, we enrolled 14 overweight volunteers with endothelial dysfunction. Each volunteer received in a random order autologous 3-day stored blood (not hemolyzed), 40-day stored autologous blood (containing up to 1% hemolyzed blood, 40-day storage), and 40-day stored autologous blood, transfused while the volunteer breathed 80 ppm NO. Transfusion with 40-day stored blood caused an increase in plasma Hb (**Fig. 2A**), plasma NO consumption, and pulmonary artery pressure as estimated by transthoracic echocardiography (**Fig. 2B**). Plasma NO consumption strongly correlated with levels of plasma Hb (**Fig. 2C**) and with increased pulmonary artery pressure (data not shown). Breathing NO maintained plasma NO consumption at pre-transfusion levels despite the increasing plasma Hb levels and pulmonary arterial pressure (**Fig. 2B**).¹⁷

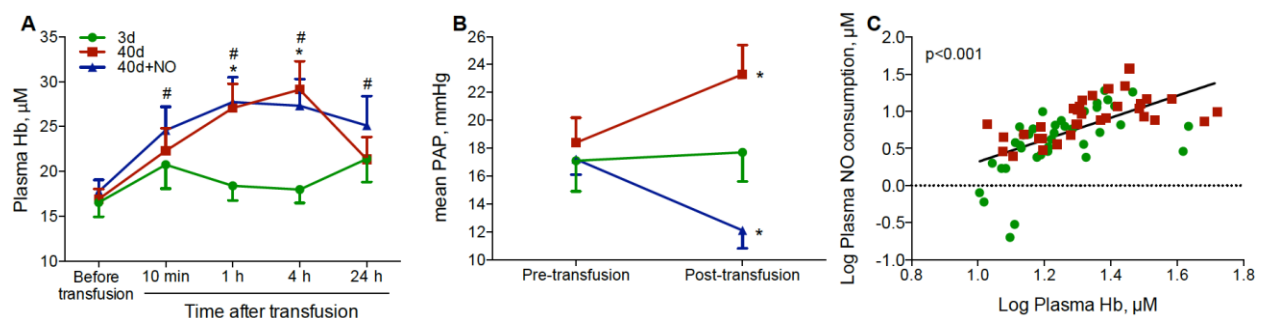
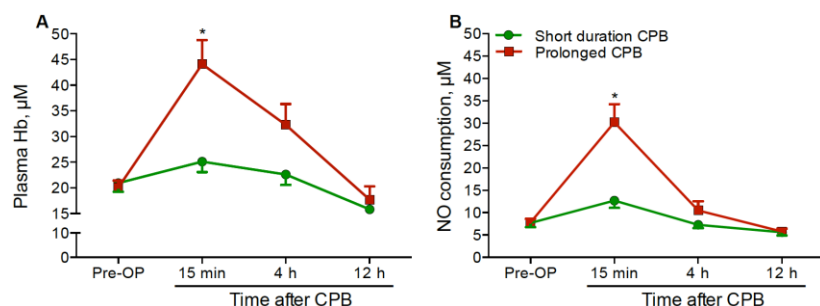


Figure 2A, B, and C. (A) Plasma Hb increased after transfusion with 40 day-stored blood (40d) * $p < 0.01$ vs. baseline (=before transfusion); 40-day stored blood transfused while the recipient inhaled NO (40d+NO) # $p < 0.01$ vs. baseline. (B) Mean PAP increased during 40d challenge ($p = 0.009$); and decreased during 40d+NO challenge ($p = 0.005$). (C) Plasma Hb concentration at baseline, 10 min and 4 hours after transfusion in volunteers receiving 3-day and 40-day stored blood correlates with NO consumption.

3) IRB #2013P000911: Prolonged CPB for cardiac surgery increases hemolysis and plasma NO

consumption. In a pilot study, we enrolled 50 MGH cardiac surgery patients who were undergoing cardiac surgery with CPB. We reported that plasma Hb (**Fig. 3A**) and NO consumption (**Fig. 3B**)



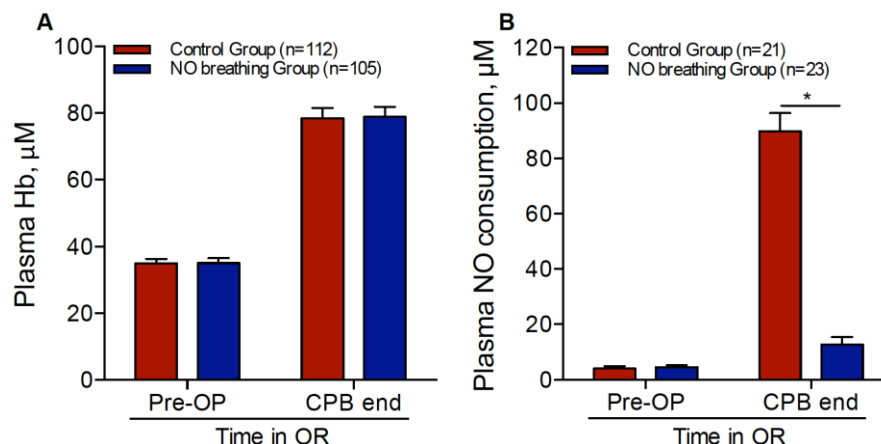
increased in patients undergoing prolonged CPB (n=25 patients with over 140 minutes of CPB) and did

not increase in patients with shorter periods of CPB (n=25, less than 140 minutes of CPB). Duration of CPB was the primary determinant of hemolysis in cardiac surgery at MGH as opposed to receiving transfused blood from a cell saver device or from the blood bank.³⁷

4) IRB #2013P002596: Administration of NO prevents acute kidney injury during prolonged CPB (over 90 minutes) in patients with no evidence of endothelial dysfunction: preliminary results of a double-blind randomized controlled trial. In a single-center, randomized, double-blind controlled trial, we compared treatment with 80 ppm NO (NO group) versus control (Nitrogen, “N₂” group). In a collaborative study performed at Xijing Hospital in Xi’an, China, we enrolled 217 adults with normal pre-op kidney function without evidence of diseases associated with endothelial dysfunction. The 217 patients underwent elective multiple valve replacement surgery with an estimated CPB time > 90 minutes. Nitric oxide gas or N₂ was administered via the oxygenator during CPB and by inhalation for 24 hours post-operatively. The aim of the study was to determine whether NO reduces AKI (primary outcome). AKI was defined as either an increase in serum creatinine by 50% within 7 days after surgery or an increase in serum creatinine by 0.3 mg/dl within 48 hours after surgery. No adverse events were associated with the administration of 80 ppm NO for 24 hours occurred. Blood Met-Hb remained below 10%. NO gas treatment decreased the incidence of AKI from 63% to 50% (p=0.04, primary endpoint achieved), as shown in **Table 1**. These results are presently being written as a manuscript for submission.

Table 1	Control (n=112)	NO (n=105)	RR or median difference (95% CI)	p
Primary Outcome, n (%)				
AKI within 1 week	71 (63)	52 (50)	0.78 (0.62–0.99)	0.04
AKI stage				
Stage 1	60 (54)	45 (44)	0.80 (0.60–1.06)	0.15
Stage 2	5 (7)	3 (4)	0.64 (0.16–2.61)	0.29
Stage 3	6 (3)	4 (2)	0.71 (0.21–2.44)	0.71
Secondary Outcomes				
RRT, n (%)	6 (5)	3 (3)	0.53 (0.14–2.08)	0.37

Plasma Hb concentration before and at the end of CPB increased to the same level in the two treatment groups (**Fig. 4A**), indicating that the extent of hemolysis was similar. However, the level of consumption of NO by plasma, in patients with high plasma Hb (>100 µM) after CPB, was 10 times greater in the group that received N₂ than in the group that received NO, as shown in (**Fig. 4B**). Thus, NO administration markedly reduces NO consumption by Oxy-Hb.



We propose to replicate the trial design we conducted in China, to investigate whether the administration of NO prevents AKI also in an MGH population with endothelial dysfunction.

c. Potential benefits to patients and/or society

Benefits to patients: The proposed therapeutic trial builds on our recent randomized clinical trial in 217 Chinese patients with valvular cardiac disease caused by rheumatic fever (IRB #2013P002596); these young patients (mean age 48 years old) did not have a history of diabetes, hypertension or obesity that are often associated with endothelial dysfunction. This study demonstrates that administration of 80 parts per million (ppm) of NO for up to 24 hours during and after cardiac surgery with prolonged CPB reduced the incidence of AKI. In other studies, in animals, we reported that endothelial dysfunction exacerbates the incidence of AKI and other organ injury after transfusion of hemolyzed blood⁶¹. This proposed single-center randomized trial at MGH will evaluate 250 patients with impaired endothelial function undergoing cardiac surgery with prolonged CPB. During bypass, we will administer NO via the bypass machine oxygenator, and, once disconnected from CPB, we will add NO for up to 24 hours to the inspiratory circuit of the ventilator to determine whether this therapy decreases the incidence of AKI in these patients.

Benefits to society: AKI and renal replacement therapy are some of the most expensive healthcare costs. One year of hemodialysis can cost up to \$72,000, while a year of peritoneal dialysis costs approximately \$53,000, according to the U.S. Renal Data System. The cost of acute renal replacement therapy in the intensive care unit in the United States is about \$3,629.80/day. Nitric oxide supplementation during and after CPB heart surgery might reduce costs associated with dialysis and improve the overall wellness of post-cardiac surgical patients.

From a scientific point of view, the results of our study will yield a new understanding of the mechanism by which exogenous NO maintains the homeostasis of vascular NO and will investigate whether

exogenous NO prevents AKI following hemolysis of prolonged CPB in patients with endothelial dysfunction. In previous animal and human studies, we showed that acute and persistent depletion of vascular NO results from the combination of two overlapping conditions: acute depletion of vascular NO by hemolysis and pre-existing (chronic) endothelial dysfunction, which renders the endothelium unable to synthesize sufficient quantities of endogenous NO. Our hypothesis with respect to the plasma bioavailability of NO is that a similar pathophysiological mechanism occurs in post-cardiac surgery patients who have pre-existing endothelial dysfunction. Endogenous vascular NO is rapidly depleted from circulating plasma by the conversion of plasma Oxy-Hb (Hb-Fe²⁺) into Met-Hb (Hb-Fe³⁺), and the impaired endothelium is unable to supplement endogenous NO production. Our study will test whether the administration of NO gas prevents the depletion of vascular NO by converting circulating Oxy-Hb to Met-Hb in patients with endothelial dysfunction.

II. SPECIFIC AIMS (Research Objectives)

- a. Specify objectives and hypotheses to be tested in the research project

Hypotheses of the proposed trial: Administration of exogenous NO will (I) decrease the incidence and severity of AKI in patients with endothelial dysfunction undergoing prolonged CPB; (II) convert free plasma circulating Oxy-Hb to Met-Hb (but not intracellular oxy-Hb) thereby preventing depletion of endothelial NO by inhibiting the ability of plasma free hemoglobin to scavenge vascular NO.

Study design: We will test these hypotheses in a randomized, double-blind clinical trial at the Massachusetts General Hospital of cardiac surgery patients with endothelial dysfunction who require prolonged CPB. Two hundred and fifty patients will be randomized to receive either supplemental NO (NO group, n=125) or nitrogen (N₂, Control group, n=125) during and after cardiac surgery for up to 24 hours post-surgery. NO will be administered via the oxygenator during CPB and by inhalation when mechanical ventilation is resumed.

Aims of the study: The primary endpoint of this therapeutic trial is to determine whether there is a difference in AKI incidence between the control group (receiving N₂) versus the study group (receiving NO). AKI is defined by KDIGO criteria as an abrupt (within 48h) reduction in kidney function correlated to an absolute increase in serum creatinine of 0.3 mg/dL or more ($\geq 26.4 \mu\text{mol/L}$) or a percentage increase in serum creatinine of 50% or more (1.5-fold from baseline) at any time during the first 7 days after surgery or, finally, a reduction in urine output with a documented oliguria of $< 0.5 \text{ ml/Kg/h}$ for $> 6\text{h}^2$.

Secondary renal endpoints include:

1. Differences in AKI severity between the two groups using the following KDIGO stages²:
 - Stage 1: Serum creatinine increase $\geq 26.5 \mu\text{mol/l}$ ($\geq 0.3 \text{ mg/dl}$) or increase to 1.5–2.0-fold from baseline or urinary output $< 0.5 \text{ ml/kg/h}$ for 6 h
 - Stage 2: Serum creatinine increase > 2.0 – 3.0 -fold from baseline or urinary output $< 0.5 \text{ ml/kg/h}$ for 12 h
 - Stage 3: Serum creatinine increase > 3.0 -fold from baseline or serum creatinine $\geq 354 \mu\text{mol/l}$ ($\geq 4.0 \text{ mg/dl}$) with an acute increase of at least $44 \mu\text{mol/l}$ (0.5 mg/dl) or urinary output $< 0.3 \text{ ml/kg/h}$ for 24 h or anuria for 12 h or need for RRT
2. Incidence and severity (KDOQI stages 3, 4, 5)³ of Chronic Kidney disease (CKD) at 1 year after surgery. CKD is defined as a reduction in glomerular filtration rate (GFR) of less than $60 \text{ mL/min/1.73 m}^2$ for at least 3 months. CKD GFR will be measured using the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation⁶⁹.

3. New requirement for RRT following AKI during hospitalization and at 6 weeks, 90 days, and 1 year after surgery.
4. Incidence of major Adverse kidney events⁴, a composite outcome of death, new RRT, and worsening renal function (defined as a 25% or greater decline in eGFR). Since a decline of 25% in eGFR may not be clinically relevant, we will also evaluate a decline of 50% in eGFR to assess for worsened renal function as part of the composite outcome at 6 weeks, and 1 year after surgery.

Secondary non-renal endpoints include:

1. Incidence of other single organ dysfunction assessed using SOFA score during ICU stay¹
2. Incidence of prolonged cardiovascular support defined as the need for vasopressors and inotropic agents, a balloon pump, or a ventricular-assist device for more than 48 hours after cardiac surgery.
3. Difference between groups of maximum hourly Vasoactive-inotropic score (VIS)^{72,73} in the first 7 days after surgery and duration of vasopressors and or inotropic agents support. VIS is calculated as Dopamine dose (mcg/kg/min) + Dobutamine dose (mcg/kg/min) + 100 x Epinephrine dose (mcg/kg/min) + 10 x Milrinone dose (mcg/kg/min) + 10,000 x Vasopressin dose (units/kg/min) + 100 x Norepinephrine dose (mcg/kg/min) + 10 x Phenylephrine dose (mcg/kg/min).
4. Difference in the duration of mechanical ventilation.
5. Difference in ICU and hospital length of stay between groups

Exploratory endpoints include:

1. Incidence and severity of AKI related to the presence of CKD at baseline, duration of CPB, duration of aortic cross-clamp, levels of free Hb, levels of NO consumption, pulmonary pressure at baseline, cardiovascular risks associated with endothelial dysfunction, scheduled procedure and EuroSCORE II.

Safety endpoints include:

1. Difference of mortality at 6 weeks, 90 days and 1 year after surgery between the two groups and stratification according to the initial EuroSCORE II.
2. Continuous Monitoring of MetHb with a non-invasive co-oximetry monitor in order to prevent methemoglobinemia (defined as MetHb > 5%)¹².
3. Continuous inhaled monitoring of Nitrogen Dioxide (NO₂) (threshold level < 5 ppm) in order to prevent dangerously high levels of NO₂.

4. Incidence of non-fatal stroke during hospitalization and at 6 weeks after surgery.
5. Incidence of peri-operative and non-perioperative non-fatal myocardial infarction, as defined by the third universal definition of MI released in 2012 by the ESC/ACCF/AHA/WHF¹⁰.
6. Incidence of post-operative bleeding calculated as the sum of blood loss through thoracic drains from the moment of closure of the chest over a period of 24 hours.
7. Differences between the two groups of transfusions with plasma and stored or autologous red blood cells (RBCs) recovered using intraoperative cell salvage devices.
8. Post-operative infections (e.g., pneumonia, wound infection, endocarditis, central line infection, urinary tract infection, sepsis).
9. Cardiac arrhythmias and other non-cardiac post-operative complications (e.g., hepatobiliary disorders, pneumothorax, pleural effusion, vascular disorders).

III. SUBJECT SELECTION

a. Inclusion and Exclusion criteria

Inclusion criteria:

1. Provide written informed consent
2. Age ≥ 18 years of age
3. Elective cardiac or aortic surgery with estimated CPB > 90 minutes
4. Clinical evidence of endothelial dysfunction assessed by a specifically designed questionnaire (Figure 3)
5. Stable pre-operative renal function without evidence of a plasma creatinine increase of ≥ 0.3 mg/dL within 3 months of study entry and without receiving renal replacement therapy (RRT)

Exclusion criteria:

1. eGFR less than 30 ml/min/1.73 m²
2. Emergent cardiac surgery
3. Life expectancy < 1 year at the time of enrollment
4. Mean pulmonary artery pressure ≥ 40 mm Hg and pulmonary vascular resistance > 4 Wood Units
5. Left ventricular ejection fraction < 30% by echocardiography obtained within three months of enrollment
6. Hemodynamic instability as defined by a systolic blood pressure < 90 mmHg
7. Administration of one or more Packed Red Blood Cell transfusion in the week prior to enrollment
8. X-ray contrast infusion less than 48h before surgery
9. Evidence of intravascular or extravascular hemolysis from any other origin:
 - i. Intravascular: Intrinsic RBC defects leading to hemolytic anemia (eg, enzyme deficiencies, hemoglobinopathies, membrane defects). Extrinsic: liver disease, hypersplenism, infections (eg, bartonella, babesia, malaria), treatment with oxidizing exogenous agents (eg, dapsone, nitrites, aniline dyes), exposure to other hemolytic agents (eg, lead, snake and spider bites), lymphocyte leukemia, autoimmune hemolytic disorders

- ii. Extravascular: Infection (e.g., clostridial sepsis, severe malaria), paroxysmal cold hemoglobinuria, cold agglutinin disease, paroxysmal nocturnal hemoglobinuria, iv infusion of Rho (D) immune globulin, iv infusion of hypotonic solutions

b. Source of subjects and recruitment methods

The study screening and recruitment will occur at the Massachusetts General Hospital (MGH) Heart Center. Each year at MGH, approximately 700 adult patients undergo cardiac surgery, with CPB lasting longer than 90 minutes. Endothelial function before cardiac surgery is not routinely assessed at our institution. However, we expect that the great majority of cardiac surgery patients are affected by pre-operative endothelial dysfunction because they have one or more risk factors, including hyperlipidemia, hypertension, atherosclerosis, diabetes, obesity and peripheral vascular diseases. Adult patients will be consented at either the outpatient pre-operative visit or in the in-patient cardiac services. Pre-operative systemic endothelial function and baseline kidney function will be evaluated before surgery. Only patients with stable kidney function and evidence of endothelial dysfunction will be enrolled. Reasons for excluding patients from the study will be noted. Stratification based on kidney function, evaluated by measurement of estimated glomerular filtration rate (eGFR)⁶⁵ calculated from the serum creatinine at the time of enrollment, will be performed as a post-hoc analysis at the end of the study.

IV. SUBJECT ENROLLMENT

a. Methods of enrollment, including procedures for patient registration

If the patient is eligible, a clinician/member of the treatment team will initially introduce the study to the patient and verbally obtain the patient's permission to be contacted by licensed physicians part of the study staff. We will recruit patients from two pools of cardiac patients for the enrollment and consent process: outpatient or admitted patients ("inpatient"). Cardiac surgeons have been presented with the objectives of the study and all have opted to be a part of this study. At MGH, cardiac surgeons require at least two visits prior to surgery, one for the initial consult and the other for a pre-admission testing area (PATA) visit closer to the anticipated surgery date. Only licensed physicians part of the study staff will approach the subject for consent during their PATA visits to participate in the trial. As for the inpatient pool, we shall screen the EPIC patient database system daily for cardiac surgical patients admitted to MGH awaiting non-emergent cardiac surgery. Should these patients meet the inclusion criteria, we will approach the patient at least a day prior to their cardiac procedure for consent. Consent will be obtained exclusively from the patient and not from a surrogate because cardiac surgery is usually a scheduled procedure. We believe it is best to obtain consent directly from the patient when he/she is fully awake. After consent is obtained, the patient will be randomized to the study group. A de-identified code will be assigned to the patient and registered on a dedicated enrollment log. After consent is obtained, the patient will be randomized to the study group. A de-identified code will be assigned to the patient and registered on a dedicated enrollment log.

b. Procedures for obtaining informed consent (including timing of consent process)

Patients will be screened in the "Cardiac surgery pre-operative clinic", MGH Cox Building floor 6 (before surgery) or in the inpatient cardiac service wards at MGH. If the patient is eligible, a clinician/member of the treatment team will initially introduce the study to the patient and verbally obtain the patient's permission to be contacted by licensed physicians part of the study staff. Only licensed physicians part of the study staff will approach the subject for consent to participate in the trial. Consent will be obtained exclusively from the patient, and not from a surrogate, because cardiac surgery is usually a scheduled procedure. We believe it is best to obtain consent directly from the patient when he/she is fully awake. After consent is obtained, the patient will be randomized to the study group. A de-identified code will be assigned to the patient and registered on a dedicated enrollment log. The signature of the consent form by the patient will be required prior to the initiation of any study procedures.

Personal Medical Information (PMI) will be accessed by the investigator(s) only for study purposes. Patients who choose not to participate in this study will receive standard care according to the procedures of the ICU, without any repercussions.

c. Treatment assignment and randomization (if applicable)

To ensure a robust and unbiased approach, randomization should account for demographic characteristics of the patients (i.e.: age, sex) and baseline estimated glomerular filtration rate (eGFR)⁶⁹. Only patients who are cared for by experienced anesthesiologists and cardiac surgeons (more than 5 years of staff experience) will participate in this study. The patients will be randomized to receive either NO (study drug) or N₂ (placebo) alone. In order to account for an elevated baseline pulmonary artery hypertension (PAH), patients with pre-operative PAH will be allocated equally to the two groups during the stratified randomization process. Given that most cardiac surgical patients do not get pre-operative right heart catheterization, and echocardiography is a poor tool for PAH, an experienced cardiac anesthesiologist will obtain PAH diagnostic parameters from a pulmonary artery catheter placed after anesthesia induction on the day of surgery. We selected a threshold of mean pulmonary arterial pressure ≥ 30 mm Hg to allocate patients to a PAH group versus low or non-PAH group. The random allocation sequence will be created using a computerized random generation program (RedCap). The randomization will be in blocks of 10 patients.

Patients will be randomly allocated to one of the test gas study groups (80 ppm NO in N₂) or the placebo group (N₂). The intervention will consist of administering the test gas via the CPB machine (into the sweep gas of the CPB oxygenator) and, after CPB via the inspiratory limb of the anesthetic or ventilator circuit and thereafter via the mechanical ventilator in the Intensive Care Unit (ICU). Test gas administration will commence at the onset of CPB and last for 24 hours. When patients are extubated they will breathe test gas via a facemask or nasal prongs. At the end of 24 hours, inhaled NO (iNO) will be weaned and discontinued while carefully monitoring hemodynamics for a period of 2-4 hours. Local guidelines for NO gas discontinuation will be followed. Using Inovent (Mallinckrodt, Ikaria Inc, N.J., USA) or commercially available tanks of Nitric Oxide (Airgas Inc, Radnor Township, Pennsylvania) or volumetrically-calibrated flowmeters, pure N₂ (placebo) or 800 ppm NO gas in N₂ will be mixed with pure O₂ or air to obtain the desired concentration of O₂ and, in the NO treatment group, a final concentration of 80 ppm NO. Nitric oxide, NO₂, and O₂ levels will be continuously monitored. Patients in the placebo (N₂) group will receive nitrogen test gas during the same 24-hour period. The inspired oxygen levels will be maintained at the usual levels required for routine post-operative care. No changes to the usual and customary standards of care for any intraoperative or post-operative treatment will be made during the study period.

d. Blinding procedure for Nitric Oxide and Nitrogen delivery

A double-blind study will be performed to avoid potential patient and investigator bias. The participants and those analyzing data and assessing the outcomes will be blinded to group assignment. The test gas tank and the gas delivery system in the operating room (OR) and at the bedside will be masked to keep the investigators and clinicians treating the patient blinded. The respiratory therapist in the ICU, the PI and a study staff will be unblinded and will prepare the appropriate test gas tanks and NO/N₂ meters. Blood Met-Hb levels and NO/NO₂ concentrations are safety concerns that the respiratory therapist and study staff will monitor and regulate (NO₂ levels will be maintained below 5 ppm, and Met-Hb below 5%, if necessary by reducing NO concentration to 40 ppm or less according to MGH guidelines NO therapy).

V. STUDY PROCEDURES

- a. Study visits and parameters to be measured (e.g., laboratory tests, x-rays, and other testing)

Screening visit: Screening will be in the “Cardiac surgery pre-operative clinic”, MGH Cox building floor 6 (before surgery) or in the inpatient cardiac service wards. Subjects will be screened if they require prolonged CPB (>90 minutes on CPB, i.e., valve replacement \pm CABG) and if the primary cardiac surgeon of the patient agrees to enroll the patient in the study.

Screening consists of:

- Review the operating room list of subjects booked for cardiac surgical procedures requiring the use of cardiopulmonary bypass with an estimated time > 90 minutes.
- Review in the Electronic Medical Chart the other inclusion/exclusion criteria including serum creatinine levels obtained as part of standard laboratory pre-surgical tests.
- Administration of a brief questionnaire to determine endothelial function with the subject verbal assent on the day of pre-surgical evaluation. The questionnaire evaluates medical history, integrating clinical and laboratory data of screened patients (**Figure 5**) and it is used as a tool to detect patients with endothelial dysfunction.
- Review the research protocol: this is a protected time for the subject to ask questions and become familiar with all aspects of the study protocol.
- Consent form: The subject will be asked to sign the consent form. The consent form will be obtained prior to the initiation of any study procedures. Only patients who are eligible, as determined after screening and assessment of endothelial function, will be asked to sign the consent form.

Should a patient be deemed ineligible and thus excluded from the study for whatever reason, including declining to participate, their reasons for exclusion will be noted. Reasons for exclusion could include but are not limited to the inclusion or exclusion criteria.

Pre-operative anesthesia management will be performed as per standardized protocols and includes the implementation of the KDIGO guidelines to reduce the risk of AKI, such as avoidance of nephrotoxic agents, discontinuation of ACEi and ARBs for the first 48 h after surgery, close monitoring of serum creatinine and urine output, avoidance of hyperglycemia for the first 72 h after surgery, avoidance of radiocontrast agents, close hemodynamic monitoring by using pulmonary artery catheter with an optimization of the volume status and hemodynamic parameters.

CABG and/or valve repair/replacement and age > 40 year old for males and > 50 year old for females and 1 out of 8 of the following criteria:	
Previous coronary artery bypass graft or PTCA (+ stent)	Yes___ No___
History or presence of intermittent claudication, critical limb ischemia, or peripheral vascular disease with the Exception of vasculitis.	Yes___ No___
History of transient ischemic attack and/or ischemic stroke	Yes___ No___
Diagnosis of diabetes (IDDM or NIDDM) requiring oral hypoglycemic agents or insulin	Yes___ No___
Hypercholesterolemia (total cholesterol > 200 mg/dl or LDL > 160 mg/dl) treated with statins, ion-exchange resins or other oral agents	Yes___ No___
BMI > 40	Yes___ No___
Hypertension (SBP 140 ≥ mmHg) treated with antihypertensive drugs	Yes___ No___
Active smoking ≥ 10 pack - years	Yes___ No___

Figure 5. Screening questionnaire to assess endothelial dysfunction.

Randomization. Patients will be randomly allocated to one of the test gas study groups (inhaled 80 parts per million (ppm) nitric oxide in nitrogen) or the placebo group (inhaled N₂), as described previously in VI.c. “Treatment assignment and randomization”.

Data collection. During the hospital stay, we will prospectively collect from the patient’s chart: hospital stay, ICU stay, duration of ventilation, amount of blood loss through chest tubes (ml), cardiac and non-cardiac post-operative complications, transfusions with plasma (units) and stored (units) or autologous (ml) red blood cells (RBCs), hospital course and mortality at 6 weeks, 90 days and 1 year after surgery stratified using EuroSCORE II.

We will also assess organ injury from the patient’s chart and laboratory data, we will calculate a SOFA score¹ and record it:

Non-fatal stroke: assessment for non-fatal stroke by the NIH Stroke Scale at baseline before surgery and at 6 weeks after surgery.

Peri-operative and non peri-operative non-fatal myocardial infarction: as defined by the third universal definition of MI released in 2012 by the ESC/ACCF/AHA/WHF (Definitions and Study Outcomes)¹⁰.

We will also collect the total time (hours:minutes) of intubation of each patient, post-operative infections, cardiac arrhythmias and non-cardiac complications, prolonged cardiovascular support as defined as the need for vasopressors, inotropic agents, balloon pump, or ventricular-assist device for more than 48 hours after cardiac surgery, maximum daily vasoactive-inotropic score (VIS)^{72,73} and duration of vasopressors and or inotropic agents support. VIS is calculated as Dopamine dose (mcg/kg/min) + Dobutamine dose (mcg/kg/min) + 100 x Epinephrine dose (mcg/kg/min) + 10 x Milrinone dose (mcg/kg/min) + 10,000 x Vasopressin dose (units/kg/min) + 100 x Norepinephrine dose (mcg/kg/min) + 10 x Phenylephrine dose (mcg/kg/min).

Renal failure will be defined according to the KDIGO criteria² and Renal Replacement therapy will be initiated according to the subject's treating physician's decision and consulting nephrologist's indication at Massachusetts General Hospital.

Blood samples collection.

Table. Summary of all Measurements and Blood Drawn for the Entire Study.

	1	2	3	4	5	6	7	8	9
	Baseline	After CPB	POD: day1	POD: day2	POD: day3	POD: day4	POD: day5	POD: day6	POD: 6 weeks
Plasma Hb, mg/dL	✓	✓	✓	✓					
NO consumption NO metabolites	✓	✓	✓	✓					
Plasma creatinine		✓	✓	✓	✓	✓	✓	✓	✓
Total Blood Drawn	6 ml	9 ml	9 ml	9 ml	3 ml	3 ml	3 ml	3 ml	3 ml

POD: post-operative day. The total amount of blood that will be collected for the purpose of this trial is: 6 ml + 9 ml + 9 ml + 9 ml + 3 ml + 3 ml + 3 ml + 3 ml + 3 ml = 48 ml of blood.

Plasma samples will be collected on the day of surgery before starting the procedure, at the end of surgery and once a day until day 7 after the cardiac procedure or until discharge if before day 7 (for a maximum of 41 mL of blood over a 7-day time period). An additional 4 ml of blood at 6 weeks after surgery will be collected, for a total of 48 mL of blood drawn for this study. In the first two days after surgery, we will measure plasma creatinine, plasma-free Hb concentration, and plasma NO consumption. From day 3 to day 7, we will measure daily plasma creatinine. At 6 weeks after surgery, we will measure creatinine. Urinary output will be recorded daily from the day of surgery to day 7.

- b. Drugs to be used (dose, method, schedule of administration, dose modifications, toxicities), include Toxicity Grading Scale (if applicable)

The intervention will consist of giving the test gas via both the CPB machine, after CPB via the anesthetic circuit, and thereafter via the mechanical ventilator. Test gas administration will commence at the onset of CPB and last for 24 hours. When patients are extubated, they will breathe test gas via a facemask or nasal prongs. At the end of 24 hours, NO will be weaned and discontinued while carefully monitoring hemodynamics for a period of 2-4 hours. Standard MGH guidelines for NO discontinuation will be adopted. Using an Inovent (Mallinckrodt, Ikaria Inc, N.J., USA) or commercially available tanks of Nitric Oxide (Airgas Inc, Radnor Township, Pennsylvania) or volumetrically-calibrated flowmeters, pure nitrogen (placebo) or 800 ppm NO gas in N₂ is mixed with pure O₂ or air to obtain a final concentration of 80 ppm NO. During CPB, the test gas is delivered through the extracorporeal oxygenator, after CPB the NO is delivered through the inspiratory limb of the anesthetic or ventilator circuit. NO, NO₂, and O₂ levels are monitored by ozone-chemiluminescence technology (Sievers Nitric Oxide Analyzer, NOA 280i, GE). Subjects in the placebo group will receive nitrogen test gas during the same 24-hour period. When patients are extubated, they will breathe test gas via a face mask or nasal prongs. The inspired oxygen levels will be maintained at the usual levels required for routine post-operative care. The test gas tank in the OR and at the bedside will be covered and blinded from the investigators and clinicians treating the patient. Only the respiratory therapist, the PI, a study staff, and the person changing the tanks and monitoring the respiratory levels of NO/NO₂ and methemoglobin will know the treatment group of the patient. No changes to the usual and customary standards of care for any intraoperative or post-operative treatments will be made during the study period. Toxicity and safety profile of Nitric Oxide administration is detailed in chapter VII.b. “Drug site effects and toxicity”.

VI. BIOSTATISTICAL ANALYSIS

a. Specific data variables being collected for the study

Pre-operative collection of patient data from the MGH electronic medical chart (EPIC) will include:

- Patient demographics
- Past and current medical and surgery history and therapy
- Co-morbidities
- Pertinent cardiopulmonary test results: i.e., echocardiography, cardiac catheterization, pulmonary function tests, and chest imaging.

Prospective collection of patient data will include:

- EuroSCORE II
- ICU and length of hospital stay
- Duration of mechanical ventilation
- Blood loss through chest tubes (ml)
- Units of plasma and stored RBCs transfused and volume (ml) of autologous RBCs transfused.
- Hospital course
- Mortality
- Organ injury (SOFA score)¹
- Non-fatal stroke, as defined by the NIH Stroke Scale at baseline before surgery and at 6 weeks after surgery
- Delirium (CAM-ICU score)⁷
- Peri-operative and non-perioperative non-fatal myocardial infarction (MI) at 28, 90 days and 1 year after surgery. MI is defined by the third universal definition of MI released in 2012 by the ESC/ACCF/AHA/WHF¹⁰
- Prolonged cardiovascular support defined as the need for vasopressors and inotropic agents, a balloon pump, or a ventricular-assist device for more than 48 hours.
- Maximum hourly Vasoactive-inotropic score (VIS)^{72,73} and duration of vasopressors and or inotropic agents support. VIS is calculated as Dopamine dose (mcg/kg/min) + Dobutamine dose (mcg/kg/min) + 100 x Epinephrine dose (mcg/kg/min) + 10 x Milrinone dose (mcg/kg/min) + 10,000 x Vasopressin dose (units/kg/min) + 100 x Norepinephrine dose (mcg/kg/min) + 10 x Phenylephrine dose (mcg/kg/min).
- Post-operative infections, cardiac arrhythmias and non-cardiac complications.

- Renal failure requiring dialysis at any time up to 1 year after surgery
- Acute renal injury, defined according to the KDIGO criteria².

b. Study endpoints

The primary endpoint of this therapeutic trial is to determine whether there is a difference in AKI incidence between the control group (receiving N₂) versus the study group (receiving NO). AKI is defined by AKIN criteria as an abrupt (within 48h) reduction in kidney function correlated to an absolute increase in serum creatinine of 0.3 mg/dL or more ($\geq 26.4 \mu\text{mol/L}$) or a percentage increase in serum creatinine of 50% or more (1.5-fold from baseline) at any time during the first 7 days after surgery or, finally, a reduction in urine output with a documented oliguria of $< 0.5 \text{ ml/Kg/h}$ for $> 6\text{h}$ ².

Secondary renal endpoints include differences in the treatment groups of AKI severity as reflected by the changes of serum creatinine and urinary output (KDIGO stages)²; incidence and severity (stage 3, 4, 5) of Chronic Kidney disease (CKD) at 1 year after surgery³; new requirement for RRT following AKI during hospitalization and at 6 weeks, 90 days and one year after surgery; incidence of Major adverse Kidney events (MAKE) at 6 weeks, and 1 year after surgery⁴; Secondary non-renal endpoints include incidence and severity of other single organ dysfunction (SOFA)¹, prolonged cardiovascular support, vasoactive-inotropic score (VIS)^{72,73}, duration of mechanical ventilation; evaluation of ICU and hospital length of stay.

Exploratory endpoints include incidence and severity of AKI related to the presence of CKD at baseline, duration of CPB, duration of aortic cross-clamp, levels of free Hb, levels of NO consumption, pulmonary pressure at baseline, cardiovascular risks associated with endothelial dysfunction, scheduled procedure and EuroSCORE II at 28 days 6 weeks, 90 days and 1 year after surgery⁹.

Finally, **safety endpoints** include: mortality at 6 weeks, 90 days and 1 year after surgery stratified according to the initial EuroSCORE II; incidence of non fatal stroke; incidence of peri-operative and non-perioperative non-fatal myocardial infarction¹⁰; incidence of post-operative bleeding and differences between the two groups of transfusions with plasma and stored or autologous red blood cells (RBCs) recovered using intraoperative cell salvage devices; Post-operative infections (e.g., pneumonia, wound infection, endocarditis, central line infection, urinary tract infection, sepsis); Cardiac arrhythmias and other non-cardiac post-operative complications (e.g., hepatobiliary disorders, pneumothorax, pleural effusion, vascular disorders); continuous monitoring of MetHb with a non invasive co-oximetry¹² monitor in order to prevent methemoglobinemia (defined as MetHb $> 5\%$) and continuous monitoring of Nitrogen Dioxide (NO₂) in inhaled breath (threshold level $< 5 \text{ ppm}$) in order to prevent reaching dangerously high levels (goal $< 5 \text{ ppm}$).

c. Statistical methods

The primary aim of this study is to determine whether supplementation with NO decreases the incidence of AKI in patients with endothelial dysfunction. We expect to enroll a total of 250 cardiac-surgery patients requiring more than 90 minutes of CPB. Patients will be randomized as previously described in the “subject enrollment” section. The two arms will be compared for homogeneity of baseline characteristics (age, weight, sex, and eGFR)⁶⁹, intraoperative course (cardiac-surgeon/anesthesiologist, CPB time, amount of blood transfusions) and post-operative data (post-operative transfusions, adverse events directly related to surgery procedures). To examine differences between the two groups, we will use an unpaired Student’s t-test for continuous variables with a normal distribution, a Mann-Whitney U test for continuous variables that are not normally distributed and a Fisher’s exact test for dichotomous variables. Continuous variables will be described as mean±SD when normally distributed, median (IQR) when not normally distributed, or count (%) if a dichotomous variable. The change in the laboratory markers over time and between the two groups will be tested with a repeated-measures ANOVA. The incidence of AKI and post-operative adverse events after prolonged CPB will be described in terms of relative risk with significance level and confidence intervals for the two groups.

d. Power analysis

The sample size of this trial was calculated based on the primary endpoint: the reduction of AKI incidence. Despite not having tracked the incidence, AKI is very common in our (MGH) patients after open-heart surgery, and it is reasonable to assume that AKI incidence is similar to those recent reports from US and European studies at major academic centers CPB (AKI incidence has been reported to be between 50 to 60%). In our prior trial on NO in cardiac surgery, the sample size was calculated to find a 30% reduction in the incidence of AKI (n=212 in that trial). We estimate a greater (35%) reduction in the incidence of AKI because we anticipate that this population with endothelial dysfunction will be more affected by the beneficial therapeutic properties of NO. Thus, in the NO group, the incidence of AKI is expected to decrease from 55% to 35.75%. The sample size needed to detect a difference, assuming an alpha error of 0.05 (two-sided test) and a study power of 0.8, is 114 patients per group. In order to account for possible dropouts, we increased our sample size by 10%. We will enroll 250 patients, 125 in the NO group and 125 patients in the control (N₂) group.

VII. RISKS AND DISCOMFORTS

- a. Procedure-related risks for this study: vein punctures and NO treatment

IV punctures. Risks related to IV punctures include hematoma formation and phlebitis. To minimize risks and discomfort, we will obtain blood from arterial lines, peripheral intravenous catheters, or central venous catheters when present.

- b. Drug side effects and toxicities:

Nitric Oxide (NO).

Risks of breathing NO at 80ppm for 24 hours are low. The theoretical risks of NO breathing include the following: pulmonary edema (especially in CREST), methemoglobinemia, hypoxia, and hypotension. In the study in neonates breathing NO for up to 4 days, study subjects had hematuria (6%), hyperglycemia (7%), sepsis (2%), infection (3%), stridor (3%) and hypotension (10%). They all occurred in the placebo group as well and none of these findings was significantly higher in the NO group. In a small study of healthy volunteers, Frostell et al reported that there were no adverse clinical events in the group inhaling NO. Of note, the average baseline methemoglobin level prior to NO administration was 0.61% and this increased to 0.77% after 10 minutes of inhalation of NO at 80ppm. This was a statistically significant, although clinically irrelevant, increase in methemoglobin levels⁶⁴. In our recent study (IRB #2013P002596), there were no side effects or drug toxicity associated with the use of NO at 80 ppm for up to 24 hours.

The inhalation of NO will be administered and monitored by a trained RRT. There will be dedicated therapists who also administer NO as part of their clinical responsibilities. They will be familiarized with the technical aspects of this protocol and supervised by one of the investigators (Dr. Lorenzo Berra), who has significant experience with NO administration both clinically and as part of investigational protocols. In addition, NO breathing is a treatment that is very common in C-ICU at Blake 8. All personnel (MDs, RTs, and RNs) are familiar with this medication. On average 5 to 10 patients are treated each month with NO breathing or similar pulmonary vasodilator in C-ICU at MGH. All study subjects receiving study gas (NO or N₂) will have continuous pulse oximetry monitoring, an RRT, and a licensed physician will be available for their care. The system for the inhalation of NO continuously monitors the NO, nitrogen dioxide (NO₂) and oxygen concentration of the inhaled gas and is stopped immediately if the concentration of NO increases beyond 80 ppm (clinically accepted upper limit). The respiratory therapist will continuously monitor the oxygen saturation via non-invasive oximetry during the procedure. Methemoglobin levels will be assessed using a non-invasive continuous co-oximetry monitor. If the

methemoglobin level rises more than 5%, NO will be halved to 40 ppm and closely monitored until a reduction occurs. If methemoglobin persists at > 5%, NO will be halved until methemoglobin is < 5%. Any study subject who experiences a side effect that is felt to be related to the study drug will not be allowed to continue in the protocol.

c. Psychosocial risks

We do not anticipate psychosocial risks to the study subjects from participation in this protocol. Each patient will personally sign the consent form before surgery, we will not ask for a surrogate's permission to enter a patient into the study. The consent form will be obtained prior to the initiation of any study procedures, including those done for screening.

Strict confidentiality will be maintained by the research team at all times, including keeping all data in a secure, locked cabinet with limited access. All specimens will be coded after they are obtained and the code key will be kept in a locked cabinet.

d. Radiation risks

Not applicable.

VIII. Potential benefits to participating individuals and to society

Potential benefits to participating individuals and to society have been described in chapter I.c.

In summary:

- a. **Benefits to participating individuals:** subjects breathing nitric oxide might benefit from a decreased occurrence of AKI after surgery and a faster recovery of the cardio-pulmonary function after CPB.
- b. **Benefits to society:** There is no drug available that has been shown to decrease AKI incidence after prolonged CPB. If the administration of NO in this trial showed clinical benefits in patients with endothelial dysfunction, other drugs/treatments could be studied to target the NO pathway for the prevention of AKI and other organ injury after CPB.

IX. MONITORING AND QUALITY ASSURANCE

a. Independent monitoring of source data

Informed consent forms, case report forms, and data will be reviewed by the principal investigator following enrollment of every 5 subjects.

b. Safety monitoring (e.g., Data Safety Monitoring Board, etc.)

An interim analysis by a Data Safety Management Board (DSMB) comprised of 3 members is planned at reaching 50% of the study population (125 patients enrolled) to ensure the safety of the study subjects. The safety data that will be reviewed includes: patient-, nurse-, research-assistant, or investigator-reported adverse events such as reason for early termination of the protocol or adverse reaction to NO. Other data to be reviewed include appropriate handling and processing of blood samples and maintenance of patient confidentiality throughout the study. The study will only be stopped if the interim analysis detects a significant increase in mortality, AKI, need for RRT, MI, post-operative hemorrhage, other significant morbidity in the NO test gas group.

Members of the Data Safety Management Board (DSMB) consist of an anesthesiologist with clinical expertise in gas therapeutics, a cardiologist and a nephrologist. Names and contacts of the DSMB members are:

- o Michael Fitzsimons, MD (Site DSMB Member). Director Cardiac Anesthesia, Anesthesia Department, Massachusetts General Hospital, Boston, MA
- o Francesco Nordio, PhD (DSMB Independent Statistician). Lead Biostatistician, TIMI Study Group, Brigham Women's Hospital, Boston, MA
- o John Prowle, MA MB BChir MSc MD FRCP FFICM (DSMB Chair). Clinical reader in Critical Care Nephrology in the Critical Care and Peri-operative Medicine Research Group at William Harvey Research Institute, Queen Mary University of London, London, UK.

c. Outcomes monitoring

Blood and urine samples will be collected until day 7 after CPB or until discharge from the hospital if this occurs before day 7 after CPB. Follow-up will continue at 30 days, 90 days, and 1 year after surgery as described in chapter II. "Specific aims" and V. "Study procedure".

d. Adverse event reporting guidelines

In accordance with PHRC policy on Adverse Event Reporting and Unanticipated Problems Involving Risks to Subjects, the Principal Investigator (Lorenzo Berra, MD) will report adverse events or other unanticipated problems to the DSMB and to the PHRC within 5 working days/7 calendar days of the date the investigator first becomes aware of the problems. Mild or moderate adverse events will be presented in progress reports at continuing reviews.

Stopping rules. The review and decision regarding altering or stopping the protocol will be performed by the principal investigator together with the DSMB. Mild or moderate adverse events will be presented in progress reports at continuing reviews. Protocol exit criteria will be:

- a. Acute worsening hypotension: decrease in mean blood pressure of > 20 mmHg not attributable to other causes, such as: hypovolemia, hemorrhage, sepsis, and acute heart failure.
- b. Sudden worsening of hypoxemia: decrease of $\text{SatO}_2 < 80\%$ at 100 FiO_2 not attributable to other causes, such as: pulmonary edema, ARDS, pulmonary embolism.
- c. An increase in NO_2 levels > 5 ppm from baseline.

Or any life-threatening symptom potentially attributed to NO administration by the physician investigator.

X. References

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Supplementary Methods

Nitric Oxide Delivery

Commercially available tanks of medical-grade NO and N₂ gas that were indistinguishable in appearance from each other were used in the trial. These gases were administered from the start of CPB and continued for a period of 24 hours. During CPB using the Stockert S5 heart-lung machine (LivaNova, Mirandola, Italy), the gases were administered through the sweep gas of the CPB oxygenator. Volumetrically calibrated flowmeters were used to administer pure N₂ in the control arm and a mixture of NO in N₂ in the NO arm. O₂ was then added to reach the desired O₂ concentration. Once CPB was completed and ventilation was initiated, the same gasses were administered through the inspiratory limb of the ventilator. A medical gas blender was used to regulate the NO concentration. After extubation, the test gas was delivered by a high-flow nasal cannula. During the delivery of NO, the concentrations of NO and NO₂ were assessed using an inline NO/NO₂ sensor (Alphasense, Great Notley, Essex, UK).

Supplementary Table 1: Surgical Features of the Study Population

	Total (N=250)	NO (N=125)	Control (N=125)	SMD
Type of Surgery				
Valve Surgery Only, n (%)	112 (44.8%)	55 (44.0%)	57 (45.6%)	0.03
CABG Only, n (%)	49 (19.6%)	25 (20.0%)	24 (19.2%)	0.02
Combined CABG and Valve Surgery, n (%)	61 (24.4%)	29 (23.2%)	32 (25.6%)	0.06
Complicates AVR, n (%)	6 (2.4%)	3 (2.4%)	3 (2.4%)	<0.001
Bentall Procedure, n (%)	17 (6.8%)	12 (9.6%)	5 (4.0%)	0.22
Other, n (%)	14 (5.6%)	7 (5.6%)	7 (5.6%)	<0.001
MAZE, n (%)	40 (16.0%)	23 (18.4%)	17 (13.6%)	0.13
Septal Defect Repair, n (%)	8 (3.2%)	6 (4.8%)	2 (1.6%)	0.18
Ascending Aorta Aneurysm Repair, n (%)	29 (11.6%)	16 (12.8%)	13 (10.4%)	0.08
LAAA, n (%)	45 (18.0%)	27 (21.6%)	18 (14.4%)	0.19
CABG: Number of Distal Anastomoses	3 (2-4)	3 (2-4)	3 (2-4)	0.128
CBP Time, min	152 (129-187)	151 (127-187)	153 (129-185)	0.014
Aortic Clamp Time, min	111 (86-142.5)	109 (85-143)	113 (90-142)	0.023
Valve Replacement				
Mitral, n (%)	67 (26.8%)	32 (25.6%)	35 (28.0%)	0.05
Tricuspid, n (%)	5 (2.0%)	1 (0.8%)	4 (3.2%)	0.17
Aortic, n (%)	88 (35.2%)	45 (36.0%)	43 (34.4%)	0.03
Pulmonary, n (%)	2 (0.8%)	1 (0.8%)	1 (0.8%)	<0.001
Valve Repair				
Mitral, n (%)	76 (30.4%)	39 (31.2%)	37 (29.6%)	0.04
Tricuspid, n (%)	19 (7.6%)	9 (7.2%)	10 (8.0%)	0.03
Aortic, n (%)	33 (13.2%)	19 (15.2%)	14 (11.2%)	0.12
Valvular Defect				
Aortic Valve Regurgitation, n (%)	44 (17.6%)	20 (16.0%)	24 (19.2%)	0.08
Aortic Valve Stenosis, n (%)	58 (23.2%)	28 (22.4%)	30 (24.0%)	0.04
Mitral Valve Regurgitation, n (%)	97 (77.6%)	48 (38.4%)	49 (39.2%)	0.02

Mitral Valve Stenosis, n (%)	9 (3.6%)	5 (4.0%)	4 (3.2%)	0.04
Pulmonic Valve Regurgitation, n (%)	2 (0.8%)	1 (0.8%)	1 (0.8%)	<0.001
Pulmonic Valve Stenosis, n (%)	1 (0.4%)	1 (0.8%)	0 (0%)	0.13
Tricuspid Valve Regurgitation, n (%)	17 (6.8%)	7 (5.6%)	10 (8.0%)	0.1

Data are expressed as counts and percentages (%)
Abbreviations: AVR, Aortic Valve Replacement; CABP, Coronary artery bypass graft surgery; CPB, Cardiopulmonary bypass; LAAA, Left atrial appendage aneurysms; NO, Nitric Oxide; SMD, Standardized Mean Difference.

Supplementary Table 2: Subgroup Analysis Among Patient with High Mean Pulmonary Artery Pressure (30-40 mm Hg)

	Total (N=52)	NO (N=26)	Control (N=26)	Unadjusted Odds Ratio (95% CI)	Adjusted Odds Ratio (95% CI)
Acute Kidney Injury, n (%)	34 (65.4%)	16 (61.5%)	18 (69.2%)	0.71 (0.23-2.24)	0.74 (0.22-2.48)
Acute Kidney Injury Severity					
No Acute Kidney Injury, n (%)	18 (34.6%)	10 (38.5%)	8 (30.8%)	Reference	Reference
Stage 1, n (%)	25 (48.1%)	14 (53.9%)	11 (42.3%)	1.02 (0.30-3.45)	0.98 (0.28-3.52)
Stage 2, n (%)	6 (11.5%)	2 (7.7%)	4 (15.4%)	0.40 (0.06-2.77)	0.20 (0.01-2.94)
Stage 3, n (%)	3 (5.6%)	0 (0.0%)	3 (11.5%)	NA	NA

Data are expressed as counts and percentages (%)

Supplementary Table 3: Subgroup Analysis Among Patient with Low Mean Pulmonary Artery Pressure (<30 mm Hg)

	Total (N=198)	NO (N=99)	Control (N=99)	Unadjusted Odds Ratio (95% CI)	Adjusted Odds Ratio (95% CI)
Acute Kidney Injury, n (%)	75 (37.9%)	39 (39.4%)	36 (36.4%)	1.14 (0.64-2.02)	1.10 (0.60-2.00)
Acute Kidney Injury Severity					
No Acute Kidney Injury, n (%)	123 (62.1%)	60 (60.6%)	63 (63.6%)	Reference	Reference
Stage 1, n (%)	54 (27.3%)	27 (27.3%)	27 (27.3%)	1.05 (0.55-2.00)	1.05 (0.54-2.05)
Stage 2, n (%)	15 (7.6%)	9 (9.1%)	6 (6.1%)	1.58 (0.53-4.69)	1.41 (0.45-4.36)
Stage 3, n (%)	6 (3.0%)	3 (3.0%)	3 (3.0%)	1.05 (0.20-5.41)	1.40 (0.24-8.15)

Data are expressed as counts and percentages (%)

Supplementary Table 4: Subgroup Analysis Among Patient with High Duration of Aortic Clamping (≥ 111 Minutes)

	Total (N=125)	NO (N=59)	Control (N=66)	Unadjusted Odds Ratio (95% CI)	Adjusted Odds Ratio (95% CI)
Acute Kidney Injury, n (%)	62 (49.6%)	31 (52.5%)	31 (47.0%)	1.25 (0.62-2.53)	1.30 (0.63-2.69)
Acute Kidney Injury Severity					
No Acute Kidney Injury, n (%)	63 (50.4%)	28 (47.5%)	35 (53.0%)	Reference	Reference
Stage 1, n (%)	43 (34.4%)	23 (39.0%)	20 (30.3%)	1.44 (0.66-3.13)	1.47 (0.65-3.35)
Stage 2, n (%)	12 (9.6%)	5 (8.5%)	7 (10.6%)	0.89 (0.26-3.12)	0.93 (0.25-3.53)
Stage 3, n (%)	7 (5.6%)	3 (5.1%)	4 (6.1%)	0.94 (0.19-4.54)	0.97 (0.19-4.94)

Data are expressed as counts and percentages (%)

Supplementary Table 5: Subgroup Analysis Among Patient with Low Duration of Aortic Clamping (<111 Minutes)

	Total (N=121)	NO (N=63)	Control (N=58)	Unadjusted Odds Ratio (95% CI)	Adjusted Odds Ratio (95% CI)
Acute Kidney Injury, n (%)	45 (37.2%)	23 (36.5%)	22 (37.9%)	0.94 (0.45-1.96)	0.82 (0.37-1.81)
Acute Kidney Injury Severity					
No Acute Kidney Injury, n (%)	76 (62.8%)	40 (63.5%)	36 (62.1%)	Reference	Reference
Stage 1, n (%)	34 (28.1%)	17 (27.0%)	17 (29.3%)	0.90 (0.40-2.02)	0.83 (0.35-1.97)
Stage 2, n (%)	9 (7.4%)	6 (9.5%)	3 (5.2%)	1.80 (0.42-7.73)	1.22 (0.25-5.91)
Stage 3, n (%)	2 (1.7%)	0 (0.0%)	2 (3.5%)	NA	NA

Data are expressed as counts and percentages (%)

Supplementary Table 6: Subgroup Analysis Among Patient with High Duration of Cardiopulmonary Bypass (≥ 152 Minutes)

	Total (N=126)	NO (N=61)	Control (N=65)	Unadjusted Odds Ratio (95% CI)	Adjusted Odds Ratio (95% CI)
Acute Kidney Injury, n (%)	60 (47.6%)	31 (50.8%)	29 (44.6%)	1.28 (0.64-2.59)	1.36 (0.66-2.81)
Acute Kidney Injury Severity					
No Acute Kidney Injury, n (%)	66 (52.4%)	30 (49.2%)	36 (55.4%)	Reference	Reference
Stage 1, n (%)	42 (33.3%)	22 (36.1%)	20 (30.8%)	1.32 (0.61-2.87)	1.38 (0.62-3.08)
Stage 2, n (%)	12 (9.5%)	6 (9.8%)	6 (9.2%)	1.20 (0.35-4.11)	1.44 (0.38-5.51)
Stage 3, n (%)	6 (4.8%)	3 (4.9%)	3 (4.6%)	1.20 (0.23-6.39)	1.40 (0.24-8.23)

Data are expressed as counts and percentages (%)

Supplementary Table 7: Subgroup Analysis Among Patient with Low Duration of Cardiopulmonary Bypass (<152 Minutes)

	Total (N=122)	NO (N=62)	Control (N=60)	Unadjusted Odds Ratio (95% CI)	Adjusted Odds Ratio (95% CI)
Acute Kidney Injury, n (%)	48 (39.3%)	23 (37.1%)	25 (41.7%)	0.83 (0.40-1.71)	0.68 (0.31-1.50)
Acute Kidney Injury Severity					
No Acute Kidney Injury, n (%)	74 (60.7%)	39 (62.9%)	35 (58.3%)	Reference	Reference
Stage 1, n (%)	36 (29.5%)	18 (29.0%)	18 (30.0%)	0.90 (0.40-1.99)	0.73 (0.30-1.78)
Stage 2, n (%)	9 (7.4%)	5 (8.1%)	4 (6.7%)	1.22 (0.28-4.51)	1.13 (0.26-4.93)
Stage 3, n (%)	3 (2.5%)	0 (0.0%)	3 (5.0%)	NA	NA

Data are expressed as counts and percentages (%)

Supplementary Table 8: Subgroup Analysis Among Patient with High EuroScore II (≥ 1.96)

	Total (N=122)	NO (N=71)	Control (N=51)	Unadjusted Odds Ratio (95% CI)	Adjusted Odds Ratio (95% CI)
Acute Kidney Injury, n (%)	65 (53.3%)	40 (56.3%)	25 (49.0%)	1.34 (0.65-2.76)	1.67 (0.77-3.61)
Acute Kidney Injury Severity					
No Acute Kidney Injury, n (%)	57 (46.7%)	31 (43.7%)	26 (50.9%)	Reference	Reference
Stage 1, n (%)	46 (37.7%)	28 (39.4%)	18 (35.3%)	1.31 (0.59-2.87)	1.61 (0.69-3.76)
Stage 2, n (%)	14 (11.5%)	9 (12.7%)	5 (9.8%)	1.51 (0.45-5.07)	2.03 (0.56-7.35)
Stage 3, n (%)	5 (4.1%)	3 (4.2%)	2 (3.9%)	1.26 (0.20-8.11)	1.71 (0.25-11.98)

Data are expressed as counts and percentages (%)

Supplementary Table 9: Subgroup Analysis Among Patient with Low EuroScore II (<1.96)

	Total (N=120)	NO (N=49)	Control (N=71)	Unadjusted Odds Ratio (95% CI)	Adjusted Odds Ratio (95% CI)
Acute Kidney Injury, n (%)	41 (34.2%)	13 (26.5%)	28 (39.4%)	0.56 (0.25-1.23)	0.55 (0.25-1.23)
Acute Kidney Injury Severity					
No Acute Kidney Injury	79 (65.8%)	36 (73.5%)	43 (60.6%)	Reference	Reference
Stage 1, n (%)	30 (25.0%)	11 (22.5%)	19 (26.8%)	0.69 (0.29-1.64)	0.69 (0.29-1.65)
Stage 2, n (%)	7 (5.8%)	2 (4.1%)	5 (7.0%)	0.48 (0.09-2.61)	0.55 (0.10-3.13)
Stage 3, n (%)	4 (3.3%)	0 (0.0%)	4 (5.6%)	NA	NA

Data are expressed as counts and percentages (%)